

*edited by*

Gillian Einstein

# Sex and the Brain



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# **Sex and the Brain**

**edited by Gillian Einstein**

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Sex and the Brain is dedicated to three generations:

To my grandmother, Juliet Lowell, who loved to make people laugh about lust;

To my mother, Margot Lowell Einstein, who embodies the passion that undergirds deeds worth doing; and

To my son, Alexander Einstein Gopen, whose existence has provided me with some of life's most satisfying pleasures.



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A generation of students have traveled with me on the journey to learn this material, adding enormously to the adventure—from the first class at Duke in 1995, which reported, "Dr. Einstein was such a good teacher that she made us feel that she was learning the material right along with us!"—to the most recent, at the University of Toronto, who are so much more savvy about the range of human sexual behavior—it has been fabulous to explore this literature with all of you.

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Finally, my list ends and starts again with Brian Cantwell Smith, whose ineffable sensibility, pluralities, and love enrich my life and dreams, convictions, and actions.



## Preface

### Development of the Reader

This compilation of papers was developed in the belief that students should read original science. It was organized in such a way as to give voice to the multiplicity of understandings within the field of hormones and behavior and the study of sex differences of the central nervous system. The small seed planted in the early days of Beach and Young has grown into a giant tree that links body and brain through studies in the fields of behavior, endocrinology, biochemistry and molecular biology of steroid hormones, neurophysiology, neuroanatomy, and neuroendocrinology. It is a wide-branching tree that stands as an example of how science grows and diversifies but all the time is firmly rooted in the soil of human curiosity. If one starts at the roots, travels the trunk, and follows each branch to its full extent, eventually a story that links body and brain, female and male, is revealed.

The chapters in this reader were originally collected for a course that I first taught in 1995, offered jointly through the Departments of Zoology and of Neurobiology at Duke University, "Sex and the Brain: The Science of Gender." It was not a standard hormones and behavior course because, at that time, I did not know about the field of hormones and behavior. I trained in neuroanatomy, not psychology; my expertise is in systems and cellular neuroscience in the areas of vision, aging, memory, and Alzheimer's disease. Embarrassing as it is to admit—I only learned that there was a whole field called hormones and behavior after I put the papers together. So the perspective of the course was from the brain out into behavior.

So, why did I develop the course?

To be honest, putting the course together was a political move. My appointment at Duke was in the Faculty of Medicine but I was also on the advisory board of the Women's Studies Program—their token scientist—and I got it into my head that it would be doing something important for women in the sciences to put together a course whose content was appropriate for cross-listing with zoology and women's studies and

that would teach substantive biology. Courses covering the feminist critique of science or the history of women in science were already plentiful. I wanted to devise a course in which, through learning some aspect of the brain, the material itself would naturally lead to questions about sex, gender, and the cultural assumptions underlying the design of the experiments.

Thus, although these readings certainly teach students about estrogens, behavioral paradigms, sexual differentiation, the hypothalamic-pituitary axis and the regions of the brain that mediate aspects of sex, they also stretch students to judge experimental design, assumptions underlying experiments, the data, and the interpretation of the data. That is what I have tried to convey in the introductions to parts I through V.

In the more than ten years of teaching these papers it has been my experience that right from the beginning students are engaged by the material. While at first curious as to why they are being assigned papers more than five years old in a science course, they shortly understand how the papers build on each other conceptually and how, by reading the foundational papers, they are seeing core assumptions of a field being worked out. This is exciting to them. They are being let in on what has always been hidden from them: the human working out of what eventually gets presented in textbooks as fact. By reading these papers, they learn that science, like every other discipline, is knowledge in the making. Would students appreciate reading original and old papers in other fields of biology? Would they get as big a charge out of reading Linus Pauling's papers working out the structure of DNA or Watson and Crick's paper trumping Pauling's model? Possibly. But for teaching an awareness of how science progresses the papers on hormones and behavior have two major advantages over the papers in other fields:

1. The field is beautifully coherent (saying a lot about the collegiality within the field); and
2. The subject matter is sex, a topic that speaks directly to the students' own struggles and engagements on the topic.

## Choice of Inclusion

In turning the course into a reader choices had to be made as to how the story should be developed and by which papers. Choosing which papers to include was, indeed, difficult. Being more knowledgeable now than in 1995 it is now apparent to me that there are some splendid classic papers that are not in this compilation. Likewise, this reader contains some very odd papers. As well, there are some papers with data that were ultimately not replicable, while missing are the papers that later did replicate some initially controversial findings. And, of course, the field is growing, so every day papers appear that should be included. Thus, out of all the beautiful work that is in the field, how did I choose these papers?

In putting this reader together, foremost in my mind was telling a story. The story is the tale we tell when we interpret a paper on sex differences in the brains of gay and straight males or differences in language areas of females and males or books on men being from one planet and women being from another. The story I wanted to tell was that of the science purported to back all that up. If a paper contributed to the flow of that story, it made its way in. Didactically it was important to give representation to how different methods can be used to answer the same question. Physiological or behavioral experiments studying the functional significance of an anatomical finding are included. Papers that allow the comparison of the primate case with nonprimate models are also included. Some papers are included to give a nod to a team that was first to publish a report on a topic. Some review articles are included to keep the story moving. On the basis of these criteria, the reader includes papers by Harris, Beach, Goy, Phoenix, Gorski, Toran-Allerand, McEwen, Kimura, Arnold, Swaab, Pfaff, O'Malley, and Meany, along with many of their students. Some surprising works by researchers from other fields also appear: Raisman, Goldman-Rakic, LeVay, and Merzenich. All of this makes a very rich blend of perspectives, approaches, methods, and findings.

In the end, the only reason papers were excluded was because the students do not have time to read any more!

## Organization of the Reader

The overarching organization of the papers is not chronological. It starts with the question of what is a dimorphism, what are the behavioral observations, and how is it that the brain is an endocrine organ. It then moves to theories on how dimorphisms are established and how and where estrogens act. After that come the experiments to understand the relation be-

tween behavior and the brain, ultimately moving to papers on sexualities and gender identification—aspects of our selves. This compilation of papers poses those questions by having five major sections that build from background concepts to the early experiments establishing the organizational/activational hypothesis, from experimental models to humans, and from molecules to mind. Papers that address traits constitutive of personhood—cognition, gay/straight, and transsexual differences—do not appear until the last section because most would agree these papers are the most speculative and sensational.<sup>1</sup>

Each of the five sections has a number of subsections comprising papers relating to each other within the subtheme. Often papers in the subsections will juxtapose rodent models, primates, and, where possible, human experiments to highlight differences between rodents and humans. The five thematic sections are background; central nervous system dimorphisms; mechanisms for creating dimorphisms; dimorphisms and cognition; and dimorphisms and identity. Included in this collection is also an epilogue, which is by Beach, himself, describing the history of the field.

Each section has an introduction discussing key concepts covered in that section, explaining the reasons for the particular grouping of papers, how the papers relate to each other, what each paper explores, and some questions students might ask while they are reading.

## Use of the Reader

My own use of this collection was as follows. Each week students would read a set of papers addressing one overarching topic within the development of the field or the underlying biology. These readings were juxtaposed with readings in popular press books on sex differences. They were chosen to create a point/counterpoint in the reductionist/pluralist debate. *Myths of Gender* or *Sexing the Body* (Fausto-Sterling) were assigned with the *Sexual Brain* (LeVay). Each week, students wrote two- to three-page commentaries on the readings to each other and responded to those commentaries also, to each other. After the first few weeks, which were spent making sure everyone had the same background, the students took over presenting the papers. Some years I gave a midterm quiz and other years, not. The final project was always a paper of no more than twenty pages in length dealing with a critique of the literature on sex differences in mental states, neuropsychiatric disorders, steroid biochemistry, or the molecular actions of estrogens.

Throughout the course, students were encouraged to think about (i) how the field developed; (ii) what the first observations were; (iii) what the diversity of

opinions on sex differences is—whether these differences are dimorphic; (iv) what the evidence for and against the organizational and activational hypothesis is; (v) what the functional relevance of anatomical differences is; (vi) why the situation is more complicated in humans than in rodents; (vii) what the nature of the field is such that so many disciplines are represented? Reading the original papers allows questions such as these to flow. Always interesting, by opening up these questions students learn quite a lot of biology; by the end they know about the biochemistry of steroid hormones, molecular and cellular actions of estrogens, physiology of single cells, anatomy of the sexual brain, and how the endocrine system mediates many behaviors.

If a class is mixed with biology, philosophy and/or women's studies students there will certainly be the need to fill in gaps in students' understanding and provide current understandings of the science covered in the papers. This can be accomplished in the first three weeks of class, after which students begin to see the same principles repeated because of conceptual overlap between papers. The reader can be used as a primary text, augmented by popular press books like Simon LeVay's *The Sexual Brain* and/or Anne Fausto-Sterling's *Sexing the Body*. Alternatively, additional texts could be neuroscience texts or one of the major texts in hormones and behavior. First-person accounts on being transgendered, the David Reimer case, or searching for the "gay gene" open the door to wide-ranging discussion. For a women's studies course, assigning these papers with any book on gender (e.g., Judith Butler's *Gender Trouble* or Anne Fausto-Sterling's *Myths of Gender*) or books questioning the science of difference (e.g., *The Mismeasure of Man*; *The Mismeasure of Woman*; *The Mismeasure of Desire*) could also be useful didactically.

Whether or not this collection is matched with supplemental texts, it can be used to teach students that a scientific paper can be analyzed from a number of often independent perspectives: the design of the experiment, the data, and the interpretation of the data. With adequate discussion, exposure to these papers will give students an appreciation of this beautiful field and enable them to judge science independently—especially the current science in this area that is appearing on the front pages of the newspapers everyday either because public figures use it to support their prejudices or because, as humans, we are just plain interested. It is important to know about the development of the field to understand just how far the notion of a dimorphism can be taken. Whether or not individual students go on to a career in science, fostering critical thinking and confidence in intellectual judgment is

the essence of our job as educators. These papers and this field support that goal.

#### Note

1. Since these are the papers students take the course to read, another possibility for organization is that the course could start there and then ask, "How did we get to this intellectual point?"



There are many assumptions embedded in any established scientific field that make a paper picked up and read at one point in time difficult to scrutinize and read critically. Without knowing what ideas have come before and how deeply they are lodged in the formulation of an experiment, one can be judging the depth of a work based on the tip of the iceberg. The purpose of this section is to take you to the early days of the field in which investigators were trying to put a quantifiable spin on the intuition that females and males are different; but how?—and give you the understanding necessary to unpack the assumptions from which papers in subsequent sections build. Papers in Part I take you to the earliest or the most elegant experiments that contributed to these understandings.

A paper purporting to show different activation patterns in the brains of females and males with a similar activation in the pattern of gay males and females is secure in this paradigm based on the following assumptions:

1. Anatomical differences represent functional differences;
2. Hormones effect behavior;
3. The brain and other endocrine organs are connected via the hypothalamus—an organ that is part nervous tissue and part gland;
4. Steroid hormones shape the development of the brain in a period shortly before and just after birth, called the “critical period”;
5. Chromosomal sex, phenotypic sex and gender identification operate via separate mechanisms;
6. Hormones act on behavior via gene transcription;
7. Males have two times of life during which there are high circulating levels of androgens—the critical period and adolescence;
8. In order to make a “male” brain, circuits need to be both organized and “defeminized.”

The selections in this section will provide a familiarity with these concepts and are grouped together in order to

1. Introduce the concept of sexual dimorphism and how it was wrestled with and formulated over a number of years, starting with Beach;
2. Establish an understanding of the intimate relationship between the hypothalamus and the pituitary through the elegant experiments of Geoffrey Harris;
3. Explain key mechanisms underlying sexual differentiation;
4. Demonstrate how chromosomal sex, phenotype, and gender identification do not have to align; and
5. Formulate an understanding of a key hypothesis in sexual differentiation: that there are organizational and activational events triggered by hormone action.

### The Concept of Sexual Dimorphisms

Before embarking on the journey of reading papers purporting to show sex differences, let’s lay out what is meant when we use the expression. “Dimorphism” means “of two forms,” an interesting formulation of sexual display in all its variations. It means that there are two forms of behavior, two forms of what things look like, two mechanisms underlying those forms: female and male. XX and XY. Lordosis and mounting. Estrogens and Androgens. This is a key concept in this field and underlies the interpretation of the science—that there are, indeed, two distinct forms. It is kind of a digital notion of all the components of sex. As is apparent in the Goy and McEwen reviews and in the original papers from the father of the field, Frank Beach, even when the early experiments were written up, things were not so on/off as the term would suggest.

Goy and McEwen’s book is important not only because it reviews the early literature in an extremely lucid and thorough manner but also serves as a pivot on which the field began to turn—a kind of “let’s look backward before we go forward” or sum up before changing paths. Experiments related in their book are about behavioral differences, how the brain might mediate those differences, and how steroid hormones shape the brain during early development as well as at other key times of life, such as puberty. After the

publication of their review, the field began turning more toward considering complex behavioral differences such as choice of sexual partner, the molecular biology of steroid hormone signaling, and differences in whole brain patterns of activation as revealed by in vivo imaging. In these later studies, however, what seemed rather plastic and malleable in earlier studies becomes hardened into two forms.

In their very complete review of experiments, behaviors, and species differences, Goy and McEwen demonstrate clearly that there are many ways to go about making sex. What seems a female behavior in some species is a male behavior in others. What is seen to depend on chromosomal components can be regulated by steroid hormones—and regulated in a fashion that places some animals in between what are commonly believed to be two distinct sexes. In their very complete review of the early literature across species, Goy and McEwen are steadfast in comparing rats to primates as well as rats to other rodents. By engaging in the comparisons across species, they make the very strong point that rats are not monkeys; monkeys are not humans.

Beach's papers take us back to the original findings of differences in behaviors and speculation that these behavioral differences might have a basis in the brain. This seems like a concept that needs no explaining to the contemporary reader but in Beach's day, this was a leap: there had been sparse, if any, direct correlation between sexual behaviors and brain structures until, in 1937, when Beach published "The Neural Basis of Innate Behavior. I. Effects of Cortical Lesions upon the Maternal Behavior Pattern in the Rat." In 1948, he solidified his invention of the field of behavioral endocrinology by the publication of his now classic book *Hormones and Behavior*, the title by which the field is now known. Just as Goy and McEwen's review leans on the idea that there is more variation and malleability in primates as opposed to the more stereotypical behavior of rodents, Beach's work is surprising in the individuality he observes in rodents. He makes abundantly clear a perception that has been lost in the literature—that just like primate behavior, rodent behavior depends on environment and circumstances. Beach observes and reports female rats entering into the stereotypical male sexual behavior, mounting. He also describes males enacting the stereotypical female behavior, lordosis. He sees these variations not as deviant but as behaviors on a continuum for both sexes. Beach's work astonishes in its openness to behavioral variations and reminds us that the best scientists are observers who are ready to see things they do not predict.

Read these chapters for their description of the definition of "sexual dimorphism," the explanation of the organizational and activational hypotheses, the neces-

sity for both masculinization and defeminization, and their centrality to the idea of sexual dimorphisms, the "sex-typical" behaviors that are used as assays for brain differences, and the design of experiments to test the stability of the notion of two forms.

### The Hypothalamic-Pituitary Axis

At the same time that Beach was working, Geoffrey Harris discovered that the pituitary is under the control of the hypothalamus. In a series of elegant and groundbreaking experiments, he documented the intimate connection via the blood supply of the anterior pituitary and the hypothalamus. He showed that nerve fibers from the hypothalamus released humoral substances into the capillaries of the primary plexus of the median eminence which connected to the portal vessels ultimately to release these substances in the anterior pituitary.

This work established the field of neuroendocrinology and cemented the connection between the brain and the reproductive system. From here it was a small conceptual leap to look for clusters of neurons in the brain that were sensitive to the influences of hormones and, which in turn, would influence the release of more hormones to regulate other endocrine organs via the pituitary. This work identified the brain as a member of the endocrine system as well as the nervous system.

The first selection in this section is a synopsis of Harris's establishment of and contributions to the field of neuroendocrinology by an anatomist who went on to do elegant experiments himself, using some of Harris' model, Geoffrey Raisman (see part II for Raisman's contribution). The other selections in this section are by Harris, himself, presenting a number of experiments that detail the connection of the hypothalamus and the anterior pituitary.

Read these chapters for the elegance of an experimental design that makes use of physiology, anatomy, and developmental biology to tell an unfolding story. Harris is a physiologist in the broadest sense of the discipline, taking into account whole body systems, using their function as the assay or test of his hypothesis. Think about the fact that if there is no circulating testosterone, males can develop a pattern of cyclic hormonal release. Note that there is no ovulation if the brain and pituitary are disconnected. This finding sets the stage for the understanding that there is a reverberating circuit encompassing the brain, pituitary, and reproductive system.

### Sexual Differentiation

The next step in the history of this story was to ask: if female and male sexual behaviors differ, and behavior

is mediated by the brain via the endocrine system, “how does it get that way?” How does the brain differentiate into one of two sexes? One hypothesis is that just as the body has a phenotype, so does the brain and as internal tubing, gonads, and endocrine organs move in a male or a female direction, so does the brain. Perhaps hormones, circulating early in embryonic development, move the fetal nervous system toward a female or a male phenotype. The selections in this section outline and build on that hypothesis.

The review article by MacLusky and Naftolin provides the insight of two longtime researchers in neuroendocrinology. The paper not only outlines what was known about sexual differentiation of the nervous system circa 1981 but presents key concepts such as “organizational and activational hypotheses,” time frames for different aspects of sexual differentiation, steroid hormone structure and action, and the “estrogen protection” hypothesis. It’s an elegant story and one that’s worth reading about in its entirety. This is the story that underlies all future studies of sex differences.

The article by Sinclair and colleagues lays out the molecular biology of the testosterone switch and the discovery of SRY as the region on the short arm of the Y chromosome that turns on the differentiation of the testes and hence, the synthesis of testosterone. When there is no SRY, there are no testes and this most often leads to a phenotypic female. Haqq and colleagues take this discovery one step further to demonstrate that the expression of SRY leads to the activation of Mullerian inhibiting substance, or MIS, whose secretion is necessary for the regression of the Mullerian ducts and the development of the Wolffian ducts, or the male internal tubing.

Read these selections for their development of the steps involved in sexual differentiation. Note that the focus is on what happens to make a male phenotype with the female phenotype depicted as a passive outcome. It is interesting to note that, at the time this reader goes to press, there is still no active story for the development of the female phenotype. The female phenotype is conceptualized as a default pathway—what happens in the absence of testosterone. There is also an emphasis on two forms only emerging from the presence or absence of SRY, which is described as a developmental switch. Note that SRY not only switches on the development of the testes but the regression of the Mullerian ducts; it not only makes the male but also destroys the female. Finally, it is interesting to compare an approach that is anatomical and biochemical to the later, molecular biological, approach. Is one more informative than the other? Does one leave room for variation while the other is conceptualized as either on or off? These are two very

different approaches and the genetic switch gains primacy.

### The Alignment of Chromosomes, Phenotype and Gender

The notion of sexual dimorphisms requires that one believe that mammals develop into only two recognizable phenotypes: female or male. To do this, however, genetics, hormone action, and rearing all must align. If any of the switches on the pathway are inoperative or turn on at an earlier or later stage, or to a greater or lesser extent, the body will not represent the types in the ways in which we expect. Because much of making two sexes is a biological process, things do not always go according to plan. In fact, the words of Hamlet to Horatio are most appropriate here:

There are more things in heaven and earth, Horatio, Than are dreamt of in your philosophy. (*Hamlet*, I, v, 166–167)

Some of the complications in the pathways for female and male begin by having an unusual compliment of chromosomes—if, for instance, the SRY portion of the Y chromosome ends up on an X chromosome, nature will have created an XX male. Others may result from defective or unexpressed genes: if after switching on SRY in an XY individual, the gene coding for the androgen receptor is absent or defective, nature will yield up an XY individual with a strikingly female phenotype (androgen-insensitivity syndrome). If all of these go according to plan but the gene for the enzyme that converts testosterone into dihydrotestosterone, 5-alpha-reductase, fails to be expressed, an XY individual will resemble an XX person until puberty, at which time, high circulating levels of androgens change the phenotype (Guevodoche). In addition, XX individuals can be born with a phenotype of their reproductive organs that is partially between female and male due to a condition of the adrenals that leads to very high production of androgens (congenital adrenal hyperplasia).

The selections in this section bother the categories of female and male by providing biological evidence that there are more than two hard and fast categories. They present cases in which the complicated path of making sex—from SRY to autosomes coding for hormone receptors to relation of hormones expressed to how people are treated—is illuminated. After reading these articles, you may question your own need to believe that humans come in only two types.

Page and colleagues report on the existence of XX males due to the exchange of terminal portions of X- and Y-chromosomal short arms. Saavedra-Castillo and colleagues suggest that phenotypic sex may be due to the involvement of sex-determining genes beyond SRY. Ahmed and colleagues describe cases of XY phenotypic females due to the absence of androgen

receptors. Imperato-McGinley and colleagues relate the now classic discovery of a cluster of XY individuals in Papua New Guinea who, because they lack the enzyme necessary to convert testosterone to dihydrotestosterone, have relatively undeveloped male external genitalia and, as a consequence, are treated as female until puberty. Finally, the Fausto-Sterling selection uses these and other examples to postulate that there may be *at least* five sexes and that we do a disservice to nature to insist on only two.

Read these selections for an understanding of how molecular biology has expanded our knowledge of biological sex and begun to blur the lines between the classic idea of two sexes. Consider the possibility they raise—that there may be genes residing on autosomes that also control sexual differentiation. Realize that, just as one can have many or few to no receptors for sex steroid action, sexual differentiation may be more analogue than often presented. Ask yourself if, just like the rest of the body, differentiation of the brain into two, distinct types might not also be less likely.

### The Biochemistry and Actions of Steroid Hormones

Although it is not critical to understand steroid biochemistry in depth to read the literature on sex differences, it is important to have a grasp of a few key concepts.

First, all steroid hormones have a common precursor, cholesterol. From cholesterol, pregnenolone is synthesized. From pregnenolone comes progesterone as well as the androgen, dehydroepiandrosterone (DHEA). Progesterone is metabolized to androstenedione which is metabolized to either testosterone (via 17-ketoreductase) or estrone (via aromatase). Estrone is metabolized to estradiol (via 17-ketoreductase). On the other pathway, DHEA is converted to androstenediol (via 17-ketoreductase), which is further metabolized to testosterone (via 3 $\beta$ -DH, delta-isomerase). Testosterone is further metabolized to dihydrotestosterone (via 5 $\alpha$ -reductase). Because each of these metabolites of cholesterol require an enzyme for conversion into the multiple forms of androgens and estrogens, it is clear that if there is any problem making the enzymes, some metabolites will not be made and other metabolic pathways will be favored. This is what happens in girls with congenital adrenal hyperplasia and boys who don't make 5 $\alpha$ -reductase (Guevedoces).

It should also be evident from understanding this biosynthetic pathway that estrogens and androgens are each a class and not a single type of steroid. There is also no production of estrogens without first producing androgens and, by the same token, if androgens are

made, they can be converted to estrogens. Thus, there is no hormone that is uniquely "female" or uniquely "male."

Second, although not exclusively, steroid hormones have their major effects by their synthesis in endocrine organs and delivery to sites of action via the blood. They acquire their specificity of action through being sequestered and amplified by receptors. Both androgen and estrogen receptors are part of a large class of steroid receptors that belong to the steroid/thyroid receptor superfamily. Other steroids like, retinoic acid, act via the same mechanisms so it is important to move away from the idea that estrogens and androgens are "sex" steroids. They can have wide-ranging effects on growth and differentiation along with the other steroids in this broader class.

Third, the receptors that sequester and amplify steroid hormone action are both classical membrane receptors and nuclear receptors. In fact, steroid hormones most commonly simply diffuse through the cell membrane because they are lipophilic. Once on the other side of the membrane, they encounter their "receptors" which are activated by binding of the steroid hormone. Once activated, the receptor-hormone complex acts at the cell nucleus where it initiates the transcription of genes and the production of protein. In other words, steroid hormone action can have very long term effects through gene transcription.

Readings in the previous chapter demonstrated that steroid hormones play a critical role in sexual differentiation. The male phenotype depends on the presence of circulating androgens both for masculinization and defeminization. It is interesting that, estrogens are available to the male brain early in development through the aromatization of testosterone to estrogen via the enzyme, aromatase. This raises the possibility that it is estrogen not testosterone itself that "masculinizes" the developing brain. It also suggests that, wherever aromatase is present, it is a region where androgens and their metabolites can act. It is also true that while most of the focus has been on androgens and estrogens, other steroid hormones can have an effect on behavior either directly or through initiating the synthesis of other hormones.

The contribution by MacLusky and colleagues takes further the idea that androgens and estrogens are not just for sex. By identifying the presence of aromatase in two areas of the brain identified with cognitive function, the neocortex and hippocampus, this article raises the very interesting possibility that "sex" steroids are not just for shaping sexual differentiation but also for shaping neural circuits for memory and volition. Later chapters in this volume will revisit the theme of the very broad actions of steroid hormones.

The other three selections all come out of the O'Malley laboratory, a preeminent site of work on the steroid/thyroid superfamily of steroid receptors. Tsai and colleagues lay the groundwork for understanding steroid hormone receptor action. Theirs is an outstanding review of the steroid/thyroid superfamily of steroid receptors detailing how steroid receptors are activated by the steroid and then affect gene transcription. This review not only lends insight into the detailed workings of steroid hormones but also places them in the broader context of molecular biology and a whole class of nuclear transcription factors, highlighting estrogen and testosterone's role not as much as sex steroids but as cell signaling factors regulating transcription.

Mani and colleagues and Law and colleagues take us back, once again, to the relation between steroid hormones and sexual behavior. In the case of these two selections, however, the steroid hormones are not estrogens and androgens. Mani and colleagues use molecular techniques to demonstrate that estrogen leads to the transcription and synthesis of progesterone which in turn leads to a sexual behavior, lordosis. When progesterone transcription is blocked, estrogen alone will not induce lordosis. Law and colleagues document how estrogen induces not just the transcription of the progesterone receptor but also the transcription of an additional twenty-one mRNAs, some of which encode metabolic enzymes, widening the role of estrogen.

Smith and colleagues detail what happens when human males do not make the estrogen receptor. It is the first report of a human who is "deaf to estrogen's call" (Natalie Angier, *New York Times*, October 25, 1994). It documents the opportune convergence of molecular science and the production of estrogen receptor knock-out mice and the appearance of the human form of the same genetic manipulation. This report also makes it abundantly clear that estrogens are not just for women or only for the reproductive system. It is a striking account of the wide-ranging effects of estrogen receptor-mediated action. It also raises the question of whether the organizational hypothesis (as it relies on the sequestration of estrogen in the body and local production in the brain via the aromatization of testosterone) can work in humans as it does in rodents since the young man in this paper reports perfectly malelike desires and behaviors.

Read these selections for their broad view of estrogens: estrogens' actions and the role of estrogen receptors. Realize that estrogens are not just for women anymore and that they have wide-ranging effects via receptor-mediated gene transcription on the entire body and possibly every body system. Finally, compare the evidence for the role of estrogens in "masculinizing" the brains of rodents with that for humans.

## Organization and Activation

The organizational and activational hypothesis has been the cornerstone of the field "hormones and behavior" ever since Beach so named the journal. One of the critical assumptions of this hypothesis is that the differentiation of sexual behavior is in tandem with the sexual differentiation of the body. Both the effects of pre- and postnatal hormones and the timing of administration had to be worked out for this hypothesis to have saliency. To implicate hormonal action on the development of the brain, behavioral endocrinologists had to first demonstrate that "sex-typical" behavior could be affected by hormones delivered during development. The earliest experiments investigated differences between pre- and postnatal administration. The most parsimonious condition was to compare the effects of androgens and no androgens on the codevelopment of external genitalia with sexual behaviors; investigations were broadened to determine whether more than sexual behavior would be affected. In the words of Young, Goy, and Phoenix, "We now know that during a period of organization and differentiation which is prenatal in the guinea pig and monkey and postnatal in the rat, the hormones act according to principles which appear to be identical with those operative during the differentiation of the genital tracts, and they effect a corresponding differentiation of neural tissues." (Young, Goy, and Phoenix, *Science* 143, p. 217.)

The first contribution by Phoenix and colleagues uses guinea pigs as the model for understanding the effects of prenatally administered hormones on tissue as well as on behavior. Interestingly, the authors develop a model of a sexual "hermaphrodite" and look to see if their behavior is "hermaphroditic as well." They conclude that the androgens have an "organizing" action on the tissues mediating sexual behavior. Because the behavior is modified, they assume that the neural substrate underlying that behavior must be "organized" as well. Young and colleagues place this previous experiment in the context of further experiments of this group, exploring the similarities between rats, guinea pigs, and primates, explaining differences between species with respect to timing of androgen administration. They also use the fullness of their professional history to extrapolate from the primate findings to contemporaneous studies and theories about human sexuality, being carried out by John Money and his colleagues.

The Meany and Steward paper investigating the dependence of a nonsexual behavior, social play, on the presence of androgens is a classic because of the elegance of the experimental design. At the time of this

experiment it was understood that estrogens aromatized from circulating androgens were actually responsible for the differentiation of the neural tissues that mediate sex-specific behaviors. Although testosterone and testosterone dipropionate were known to masculinize the genitals, in the brain that same androgen secreted by the testes, would be aromatized to estrogen. Estrogen then affected behavioral and neural change. If estrogens were administered directly during the critical period for sexual differentiation, “masculine” behaviors would develop in female rodents. Meany and Steward, however, were interested in the role of androgens directly on social play, so they administered a form of androgen that is not aromatized or they implanted blockers of aromatase. Their contribution is of particular interest as “the exception that proves the rule.” That is, it elucidates essentially the only sex-specific behavior that depends on androgens directly—and it is not associated with sex.

Read these selections for the form of the experimental design. They are meant to test a number of possible outcomes and then report on them all as subsections of a much bigger question. It is a type of experimental design that one rarely sees anymore but which was the rule in neuroscience until the mid-1980s. Read the selections also for the authors’ careful administration of androgens, estrogens, and aromatase blockers in order to rule out any overlap of function between the two hormonal systems. Note that there is a critical period for organizational effects much as there is a critical period for vision and language acquisition. Think about how the idea of activational effects undermines the static notion that there is one period during which neural circuits are established.

Finally, attend to suppositions about what is “female” and what is “male” behavior. Think back to Beach’s work that allows for behavior—even sexual—to be situational and influenced by other social cues. Note that in these papers behavior is portrayed as more stereotypical.

## Introduction

Sex differences in behavior, like sex differences in body structure, are determined by a combination of genetic, hormonal, and external environmental factors. Dissection of the relative importance of these factors has occupied a major part of the research and writing on the subject, and a discussion of these issues took much of the time during the Work Session. First, the term *sexually dimorphic* was discussed, after which the organizational hypothesis concerning the development of these behaviors in mammals and birds was outlined.

The term *dimorphism* refers to the existence of two distinct *forms* within a single species. The term *sexually dimorphic behavior*, by extension, implies two different forms of behavior exhibited by the male and the female. Some workers have accepted usage of the term to mean not two different forms of behavior, but a response shown exclusively by one sex and not by the other. While some behaviors observed under natural circumstances, e.g., the ejaculatory pattern, are present in one sex but not in the other of most species, few other kinds of behavior could be classified as sexually dimorphic by this strict definition. Under appropriate conditions of hormonal treatment and testing, for example, male rats will display receptive and lordosis behaviors, and female rats will exhibit circling and mounting behaviors. In the discussion and presentations that follow, the term sexually dimorphic does not usually indicate the presence of a given response in one sex exclusively and its absence in the other, but, rather, any measurable difference in the parameters of the response for the two sexes. Thus, for example, both males and females eat the same food, but one sex may ingest measurably more than the other, or one may eat more often than the other without actually eating more food. The running activity of rats provides a different kind of example. Both sexes run, but the temporal patterning of their locomotor activity, measured daily, is distinctly different. Again, in a given species, hypothetically at least, males and females may not differ in the amount of aggression shown but may

differ markedly with respect to the occasions or stimulus situations that evoke aggression. For example, in territorial species, males may attack primarily intruder males, and females may attack primarily intruder females. Finally, for hormonally mediated behavior, the sexes may differ only in the amount or kind of hormone normally involved in regulating the display of the behavior. Examples of such differences are to be found in the experimental induction of lordosis responses in male and female rodents when the male requires more estrogen than the female, or in the regulation of spontaneous mounting behavior, which, in some species, is facilitated by sequential estrogen and progesterone in the female and by testosterone in the male.

To describe these behavioral differences between the sexes as sexual dimorphisms does not violate common usage of the term by the morphological sciences. Among mammalian forms, both sexes have a pelvis. The difference between the sexes is not in the presence or absence of a pelvis or pelvic outlet, but in its size, girth, or other quantitative measure. Or there may be dynamic changes in the shape of the pelvis restricted to one sex and occasioned by pregnancy and parturition. Countless examples exist in morphology of sexual dimorphisms based only on quantitative differences, differences in intensity (e.g., coloration), or in response of a specific structure to hormonal stimulation. Nevertheless, despite the reasonableness of extending the term sexual dimorphism to behavioral differences, no implication is intended by the contributors concerning a structural or morphological basis for the measured dimorphism. The reader, moreover, is cautioned against drawing any such inference. Furthermore, the mere demonstration of sexual dimorphism cannot specify either its causation or its biological function. It was, in fact, the very task of the Work Session to determine, to the extent possible, the causes of a variety of described sexual dimorphisms in behavior, and for this purpose we have drawn heavily on the principles of sexual differentiation provided by embryologists, geneticists, and anatomists working with morphological sexual characteristics.

Current concepts of morphogenesis hold that the genetic sex of all vertebrates determines whether the embryonic genital ridge develops into a testis or an ovary. The means of action by which chromosomes direct the differentiation of the embryonic gonad are unknown; but it is known that the type of gonad differentiated determines by its secretory products whether male or female secondary reproductive organs develop. According to the organizational hypothesis (Phoenix et al., 1959), not only the reproductive organs but also the neural processes mediating sexual behavior in mammals have the intrinsic tendency to develop according to a female pattern of body structure and behavior. However, they pass through a restricted period when a bisexual potentiality exists in both sexes. Circulating androgens from the testes are both morphogenetic and psychogenetic. They enhance the development of male behavior, as well as the differentiation of male reproductive organs, and in both instances the modifications induced by the hormone are enduring. Up to this time, ovarian secretions have not been assigned a significant role in the differentiation of the sexual behavior or reproductive tract characteristics in mammals, and female sexual characters develop even in the complete absence of ovaries. For female behavior, the critical organizing influence seems, in fact, to be either the absence of potent androgens, or, alternatively, if they are present, their concentrations must be too low to initiate the events that lead to a masculine organization. Moreover, estrogens circulating in higher than normal concentrations not only do not promote the development of female characteristics, but in some mammalian species they may act like the androgens to enhance masculine behavioral traits. This "organizational" hypothesis addressed itself to hormones in the bloodstream and not in the cell. Information as to whether a specific androgen (e.g., like testosterone) is a prohormone that needs to be converted into another substance in the cytoplasm or nucleus of cells comprising target organs before it produces its enduring modifications is viewed as clarifying the cellular mechanism of organization rather than as contradictory to the hypothesis.

The hormonal induction of enduring effects on behaviors and morphology (i.e., the organizing action of the hormones) is restricted to a limited period of development, the so-called "critical period." Use of the "critical period" concept can be criticized on the grounds (1) that the changes induced by hormones are not identified physiologically and/or anatomically, and (2) that in the absence of such information the changes cannot be evaluated in terms of whether or not they are "critical." In preference to such loose usage of concepts, therefore, some investigators (Goy et al., 1964) have proposed the alternative term, "period of maxi-

mal susceptibility" (or sensitivity) to the actions of hormones on the tissues mediating behavior.

Regardless of the terminology, however, periods have been identified in early development when hormones can most readily effect enduring changes in the ways in which an individual is destined to behave. In placental mammals, this period of sensitivity for the developing offspring does not bear a constant relationship to the event of parturition or birth. Based strictly on empirical studies, the most effective period for modification is shortly after birth for the rat (Grady et al., 1965; Harris and Levine, 1965), the mouse (Campbell and McGill, 1970; Edwards, 1971), the hamster (Swanson, 1971), and the ferret (Baum, 1976); it is prior to birth for the guinea pig (Goy et al., 1964), the sheep (Short, 1974), and the rhesus monkey (Goy, 1966, 1968). In certain species, e.g., the dog (Beach and Kuehn, 1970; Beach et al., 1972), the relevant hormones may have to be present for some time both prior to and shortly following birth. In the few scattered studies that have been done with the rabbit, efforts to identify a maximally sensitive period have been unsuccessful. Campbell (1965) reported that a variety of steroids injected into female rabbits for a few days following birth failed to modify sexual behavior. Anderson (1970) reported that prenatal injections of androgen abolished maternal behavior of female rabbits but were without effect on adult sexual behavior, ovulation, or ability to maintain pregnancy. In view of demonstrated empirical precedents (e.g., dogs), it seems that the rabbit may also require exposure to appropriate steroids both pre- and post-natally in order to induce marked masculinization of genetic females. This problem needs to be reinvestigated.

For the limited number of species studied, there is an apparent relationship between fetal or larval opening of the eyes and the time of effective hormonal influence. In all cases, the developing organism has to be exposed to the hormone prior to eye-opening for enduring modifications to be induced. Since opening of the eye is correlated with neural maturation and development, it is relatively likely that hormones have to act on a nervous system at a specifiable stage of incomplete development in order to induce the changes of interest. While eye opening may, in a very general way, mark the end of the period of hormonal sensitivity, no specific event has been identified that serves as a marker for the beginning of this period. Considering, moreover, the extensive array of psychological functions, processes, and behavioral patterns now known to be influenced by hormones present during early stages of development, the utility of the notion of a single "period" should be questioned. Perhaps, rather, a sequence of "periods" exists, such that each step in the sequence is sensitive to organizational actions of the

hormones on only one or a few specific traits. Some evidence supporting this possibility is presented in later sections of this report and, therefore, is not repeated here; but it may be of value to point out that the basic notion is consonant with what is known regarding the differentiation of separate portions of the mammalian male reproductive tract (Burns, 1961).

The original empirical studies on guinea pigs led to the hypothesis that androgens present before birth organized the pattern of sexual behavior into the male type (Phoenix et al., 1959). In those studies, evidence was presented demonstrating not only the enhancement of behavior normally typical of the male (mounting) but also suppression of behavior normally typical of the female (estrogen-progesterone induction of lordosis). Inasmuch as the male guinea pigs studied at the time showed only weak and irregular lordosis responses to induction procedures, such fragmentary responses were considered male-typical. Therefore, when the same kind of fragmentary and weak lordosis responses were found to be characteristic of genetic females exposed to androgen prenatally, this "suppressed" form of lordosis was conceptualized as one aspect of the masculinizing action of prenatal androgen. This suppressive action of lordosis was thought to be as significant to the organization of behavioral maleness as the enhancement of male behavior itself. In this respect, then, both suppression of female characters and enhancement of male characters were subsumed under "masculinization." In the time since the original statement of the organizational hypothesis, additional studies, some involving different species, have shown that the suppression of female-typical behavior can be accomplished independently of the enhancement of male-typical behavior. These discoveries have led to the adoption of a new terminology. The term "defeminization" has been adopted and widely used to refer to hormonal effects involving the suppression of female-typical behavior in genetic females only. The term "masculinization" is now generally reserved only for hormonal effects involving the enhancement of male-typical behaviors in genetic females. For the genetic male, complementary terms of "feminization" and "demasculinization" have been brought into usage. More complete discussion of these terminological problems can be found in Beach (1971) and Goy and Goldfoot (1973).

The real advantage of the use of terms like *masculinization* and *defeminization* (or *feminization* and *demasculinization*) lies not alone in the conceptualization of these as independent processes. The use of these terms encourages questions about spontaneous bisexuality that might be overlooked with a different theoretical framework. For example, some female guinea pigs show frequent mounting behavior as well as vigorous

lordosis at the time of spontaneous or induced estrus, and these characteristics are genetically influenced (Goy and Young, 1957; Goy and Jakway, 1959). Adoption of the newer terminology readily facilitates the question: "What is the agent that masculinizes without defeminizing these females?" An alternative restatement of this question might be: "What are the conditions that impose a bisexual organization on females within a defined genome?" The summaries of discussions from the Work Session only hint at possible answers to such questions, but, at the very least, they show that the contemporary form of question avoids more of the purely semantic problems than was previously possible. While there is still reasonable and serious dispute regarding the biological cause of different organizations of sexuality and sexual behavior, hormonal hypotheses have earned a respectability that allows their inspection even for problems of human sexual behavior, a permission that was not readily granted by clinical workers a few decades ago.

As far as can be ascertained, behavioral traits that exhibit sexual dimorphism are influenced only by the gonadal hormones, regardless of whether these hormones are secreted by the gonads, the adrenals, or both. Restriction of behavioral sexual dimorphisms to gonadal hormone influence may reflect the fact that many or all of these traits are directly or indirectly related to reproductive fitness. As Nottebohm emphasizes in his discussion of avian sexual dimorphisms in a later section of this report, the functions of behavioral dimorphisms in attraction and arousal of mating partners are adaptive when their display reflects full reproductive competence (i.e., fully functional gonads).

Studies of sexually dimorphic behavior are numerous for mammalian species and much less well represented for other forms. It will not be surprising, therefore, if what has been learned about hormonal influences on such characters requires revision and extension as more data become available. Even for mammals, however, evidence clearly supports the classification of male-typical characters into three basic types in terms of their relations to the gonadal hormones. Type I encompasses those behavioral characteristics that cannot be brought to full expression unless the relevant hormone(s) is (are) present in adequate amounts in the circulation during both the critical period of early development and also during a later life stage. Some behavioral traits, in other words, require both early organizational actions and later activational actions of the hormones. It is not without interest, moreover, that the hormones that accomplish the organizational effects and those that activate the behavior at a later age are most often the same. Examples of behavioral traits that require hormonal actions both early and late in development are male

intromissive and ejaculatory behavior [...] and the male fighting behavior of some strains of mice [...]. These behavioral traits find a distinct parallel in the actions of androgens on male accessory reproductive organs like the prostate and seminal vesicles. For these structures, organizational actions during an early critical period first effect structural differentiation, and later in life activational actions induce secretory function.

A second type of relationship (Type II) characterizes those behaviors that seemingly require only activation at later ages by the appropriate hormone. For these behavioral traits either androgen is not necessary during the early critical period, or the amount of androgen normally present at that time in both sexes is sufficient for their organization. An example of this kind of trait is the yawning behavior of rhesus monkeys (Goy and Resko, 1972). This response is ordinarily displayed much more frequently by adult males than by females or juveniles of either sex (Bielert, 1978). However, its frequency of display by females or juveniles can be augmented to a level equal to or exceeding that of the normal male by administration of exogenous testosterone. The mounting behavior of some strains of rats may be another example (Whalen et al., 1969), although females that mount as adults due to exogenous testosterone may have undergone some in utero virilization by exposure to their brothers' androgens (Clemens and Coniglio, 1971). Morphological parallels exist for this type of trait as well, and the induction of the growth of facial hair and balding response of human beings are well-known examples. These traits, more common in adult males than females, can be induced in the latter by exogenous testosterone given only in adulthood.

Type II behavioral traits, which can be activated at will independently of organizational influences, are not enduring features of the individual. Such traits are manifested only during the time the activational hormone is present. When the hormone is removed by castration or declines spontaneously, the manifestation of the behavior is measurably altered, usually lessened in frequency or intensity. These traits depend entirely on concurrent hormonal levels during later ages when the behavior is normally displayed, and they differ from Type I traits not in their activational requirements but in their independence from organizational influences of the hormones during the critical period.

Type III traits, perhaps because of their more recent discovery, or perhaps because definitive evidence for them is more difficult to obtain, occur less frequently than Types I and II. These behavioral traits require only organizational actions of androgens, and no activational influence is required for their full expression by the individual. Such traits are manifested as well, or nearly as well, in males castrated prior to puberty

(but after the end of the early critical period) as in intact males. They cannot be "activated" in spayed females by administration of exogenous androgen during the postcritical period stage of development; but they can be easily induced in females by appropriate treatment with androgens during the critical period. Examples of such types of behavior are the juvenile play and mounting behavior of rhesus monkeys [...] and the micturitional patterns of the dog (Martins and Valle, 1948; Beach, 1974). No parallel morphological systems come readily to mind beyond the basic sexual differentiation of the reproductive tract tissues.

Recognition of the general and usual existence of these three types of relationships between hormones and sexually dimorphic behavior provides a perspective that renders a common mechanism of hormonal action unlikely. The complete contrast between Type II and Type III, the former operating entirely through activational mechanisms and the latter entirely through organizational mechanisms, suggests, at the very least, that the nature of the hormonal interactions with cellular machinery might contrast correspondingly.

This introduction to the problems of hormonal regulation of behavioral sexual dimorphism would be incomplete without some added information on other vertebrate classes. Birds, reptiles, amphibians, and fishes are clearly more diversified and less completely studied than mammals. In one precocial avian species, the Japanese quail, Adkins (1975) has shown that injection of fertile eggs on day 10 of incubation with either testosterone propionate or estradiol benzoate produced feminized males and normal females. Such treated males showed suppression of male sexual responses as adults and augmentation of feminine receptivity, exactly the opposite of the general effects of steroid treatment in mammals. Estradiol was clearly more potent in demasculinization and feminization than testosterone, and the latter hormone probably accomplishes its organizational effects through aromatization to estradiol or estrone. Thus, although organizational influences of hormones are demonstrable in birds, both the effective hormone and the sex affected are different from the mammalian case, and resolution of this difference has been sought in terms of the influence of heterogamety on hormonal organization. Paralleling the mammalian story, however, the period for organizing actions of estrogens in birds is prenatal in precocial and postnatal in altricial forms. Orcutt (1971), using altricial pigeons, obtained evidence for demasculinization and feminization of males treated with implants of estradiol for varying periods of time post-hatching.

Studies appropriate to the concerns of the Work Session have not been carried out in reptiles, and the information on amphibians and fishes is incomplete for present purposes. Nevertheless, frogs, toads, newts,

and salamanders can be completely sex-reversed by incubating fertilized eggs in water containing small amounts of hormone (Burns, 1961; Foote, 1964; Gallien, 1965, 1967). These sex reversals are so complete that genetic females grown in water containing testosterone develop fully functional testes, produce sperm, and mate with normal females to produce only female offspring. Conversely, in other species, males grown in water containing small amounts of estradiol are comparably reversed and will mate with normal males. Unfortunately, for these species information is lacking on specific hormone-behavior relationships in adults and on the reversibility of sexually dimorphic behavior at later ages. In short, it is not known whether critical periods exist for the organization of sexually dimorphic behaviors of any sort.

Of all the vertebrate phyla, fishes are the most diversified and least understood. Among teleosts, hermaphroditism is an extremely common occurrence. However, our search for examples that might provide evidence for organizational influences that conform to those of mammals has to exclude these spontaneously hermaphroditic forms. Complete transformation of all female offspring into males has been accomplished in *Tilapia mossambica* by treating fry with methyltestosterone for about 2 months after hatching (Clemens and Inslee, 1968). Newly hatched goldfish, treated for about the same period of time post-hatching, were transformed either to all-female broods when estrogens were used or to all-male broods when androgens were used (Yamamoto and Kajishirna, 1968). The addition of either testosterone or estradiol to the aquarium water of young cichlids resulted in feminization of males, and such feminized males could be bred to normal males (Hackmann and Reinboth, 1974). When sexual differentiation of the gonad normally occurs before birth (or hatching), treatment of fry is ineffective. For the viviparous guppy, treatment of gravid females with methyltestosterone for only 24 hours resulted in all-male broods (Dzwillo, 1962).

In general these studies of experimental sex reversal have not investigated hormonal influences at later ages, and the extent to which these same species could be sex-reversed as adults has not been carefully worked out. A loss of plasticity with maturity is certain for hormonal reversal of the gonad in some forms (Hackmann and Reinboth, 1974) and is suggested for behavior by the finding that adult female *Platygoecilius variatus* treated with methyltestosterone showed only weak and preliminary male courtship patterns (Laskowski (1953). Clearly, much more work is needed before concepts like the critical period can be meaningfully applied to fishes, and for spontaneously reversing forms such a concept is not likely ever to be applicable without modification.

Spontaneous sex reversal from functional female to functional male is well known and occurs among such diverse forms as zooplanktivores (Popper and Fishelson, 1973), cleaner wrasses (Robertson, 1972), gobies (Lassig, 1977), and parrot fish (Choat and Robertson, 1975). Though "spontaneous," once reversal has occurred, no reversal or regression to the original type has been documented for any species. Recently, Shapiro (1977) has completed an elegantly detailed study of sex reversal in the protogynous coral reef fish, *Anthias squamipinnis*. In this species all juveniles mature as females, and only some transform later into males. This social species lives in heterosexual groups, and the loss of a single male from the group is followed by a surprisingly rapid sex reversal (requiring only 1 week or so) in one of the females. The changes include transformation to the color pattern, gonadal histology, and behavior of the normal male. For each group, the transformation is limited to a single female, and the factors determining which female will undergo transformation are not entirely clear. In part, however, the transforming female is suddenly treated (i.e., behaved toward) quite differently by the other female members of the group. Well in advance of any outward physical signs of change, the nonreversing females behave toward the reversing female as though she were male. Shapiro (1977) argues that, since all-female groups occur in nature, it is the change in social behavioral patterns that are more causal to the females' sex reversal than the removal or loss of a male. This fascinating model for social environmental control of hormonal functions deserves detailed future study. The phenomenon may have parallels or even partial homologies at other phyletic levels. The opposite type of spontaneous sex reversal (from male to female) also occurs among fishes. In the anemone fish *Amphiprion*, a monogamous but group-living form, the single female (always the largest and oldest in the group) suppresses the transformation from male to female by aggressive dominance over the smaller and subordinate males (Fricke and Fricke, 1977). Only one of the males in the group, the most dominant, has fully functional testes, and in all other males testicular development is correlated with dominance status.

This brief survey of vertebrate sexuality serves only to show that no fundamental uniformity exists that is readily apparent. Phyla differ, as do species within phyla, with regard (1) to the hormone that has morphogenetic and psychogenetic potential, (2) to the genetic sex that can be more easily reversed, (3) to the state of maturation at which reversal can occur, (4) to the extent to which sex can be hormonally reversed, and (5) with regard (probably) to the role of hormones in the organization and activation of specific behaviors. Nevertheless, despite these differences, there is, as yet,

no compelling evidence against the most abstract level of generalization that would assert the possibility that both organizational and activational influences of the gonadal hormones are represented among all vertebrate phyla. The fact that both kinds of hormonal influence may not be demonstrable in every species is not, after all, a more difficult conceptual flaw than the circumstance that both influences are not always demonstrable for every type of sexually dimorphic behavior shown by a single species. Nor is it any more disconcerting, logically, than the fact that a sexual dimorphism found in one species may not be present in another, or may be present but totally reversed in a third. On the contrary, the boundless variation of behavioral and morphological sexual dimorphisms is one of the richest challenges to empirical science in general and to endocrinology and neurology in particular. In searching out mechanisms of proximate causation, we cannot afford to ignore the adaptive functions of these dimorphisms; we must be willing to entertain the possibility that some dimorphisms have neither a genetic nor a hormonal basis. The notion that selection strongly favors the complete environmental determination of sex in some species (Charnov and Bull, 1977) obliges us to tolerate a possible like determination of behavioral characters typical to each sex. For some highly social species, like the human being, culture may define the types and limits of sexual dimorphisms. Worse luck yet, the individual human being may be forced to learn or acquire those dimorphisms that, like the sexreversing *Anthias squamipinnis*, the behavior of his or her peers thrusts upon him/her.

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### Differentiation of Sexual Behavior in Rodents

Gerall provided the Work Session with an extensive background, citing many experiments demonstrating that, when fetal or altricial mammals are exposed to certain steroid hormones, they will, as adults, display heterotypical sexual behavior with higher frequency and quality than untreated subjects. Thus, the organizational action of perinatal hormones has most often been viewed as modifying the threshold of elicitation of mating and other behaviors involved in reproduction, including aggression and maternal activities. The nature of these behaviors and the degree to which they are restricted to one sex or are shared by each sex vary considerably among species. Thus, at this stage of knowledge, it has been necessary to test the implications of the organizational hypothesis with more than one species. Perinatal hormone manipulations have not yielded the same results in different species, although species differences in alteration of sexual behavior after perinatal administration of androgens to females differ primarily in degree, not in direction. The results for a wide variety of mammalian species are summarized in table 2.1. These discrepant findings have been instructive in directing attention to physiological or other variables that must be included in a general theory attempting to understand the ontogeny of reproductive behavior.

The two most frequently studied rodents are the rat and hamster. The receptivity pattern of the rat includes soliciting or proceptive behaviors (Beach, 1976), including darting, hopping, and ear wiggling, which precede and perhaps determine the occurrence of mounting by the male. The lordosis, which occurs only when skin is contacted, is relatively brief, generally lasting while the male is mounting and 1 or 2 seconds thereafter. Proceptive behaviors are not evident in the receptive hamster, and her lordosis, once assumed, is often held for longer than 5 min. Its maintenance is considerably less dependent on contact than it is in the rat. During a mating test, the male hamster not only engages in fewer preliminary behaviors but mounts less frequently than the male rat.

A consistent difference also exists between these species: the tendency to exhibit heterotypical sexual behaviors. Male hamsters more readily display female behaviors than male rats, and female rats more readily manifest male behaviors than female hamsters. Lordosis is induced by estradiol and progesterone treatment of male hamsters castrated as adults (Tiefer, 1970). A similar treatment does not induce lordosis in adult male rats. Mounting occurs spontaneously in neonatally untreated intact female rats but rarely in intact female hamsters. Administration of testosterone propionate (TP) in oil readily induces mounting in ovariectomized rats but not in hamsters (Paup et al., 1972; Whitsett and Vandenberg, 1975). Finally, a response resembling the ejaculatory pattern of males can be elicited from normal adult female rats by either prolonged estrogen treatment or by electrical stimulation in adulthood (Emery and Sachs, 1975). A similar behavior has not been observed in female hamsters.

The disparity between rat and hamster sexual behavior tendencies can be rationalized, at least in part, by arguing that hamsters are exposed to less androgen or are less responsive to circulating androgen during their critical periods of development. As a predictable consequence of either circumstance, the male hamster would have the lower threshold for lordosis and the female hamster a higher threshold for mounting than their rat counterparts.

In many rodents, a particularly important portion of the critical period for the organization of sexual processes occurs before birth. Clemens (1974) correlated the number of male rats in a litter with the propensity of the females in that litter to show mounting behavior when tested in adulthood. The probability of mounting was 0% in females from litters with 0 to 1 male, but 75% in females from litters with 4 to 5 males. Moreover, preliminary results indicated that both anogenital distance, a reliable measure of androgen exposure, and mounting behavior in adulthood were highest for females located in utero in closest proximity to males (Clemens and Coniglio, 1971). These parameters were the lowest for females located in utero distant to males. These studies, which were replicated in mice by

**Table 2.1**

Effects of perinatal administration of androgen to females on the development of sexually dimorphic traits [Gerall]

		Dimorphic Characteristic <sup>†</sup>			
Species	Critical Period*	Ovulation	Type of Sexual Behavior		Reference
			Female	Male	
Rat	Post	↓↓↓↓	↓↓↓	↑	See text
Mouse	Post	↓↓↓↓	↓↓↓	↑↑	Barraclough and Leatham, 1954 Edwards and Burge, 1971
Hamster	Post	↓	↓	↑↑↑↑	Carter et al., 1972; Paup et al., 1972, 1974; Whitsett and Vandenberg, 1975
Ferret	Post	?	0	↑↑↑	Baum, 1976
Dog	Pre + post	?	↓↓	↑↑	Beach and Kuehn, 1970; Beach et al., 1972
Guinea pig	Pre	↓↓	↓↓↓	↑↑	Phoenix et al., 1959; Goy et al., 1964; Brown-Grant and Sherwood, 1971
Sheep	Pre	↓↓	↓↓	↑↑	Short, 1974; Clarke et al., 1976b
Rhesus	Pre	Onset delayed	?	↑↑	Goy, 1970a,b

\* Pre = prenatal; post = postnatal.

† Arrows indicate direction and relative ease of obtaining effect: ↓ = defeminization; ↑ = masculinization; 0 = no effect.

Gandelman and co-workers (1977) and vom Saal and Bronson (1978), were extended in rats by Tobet, Dunlap, and Gerall,<sup>1</sup> who determined the sequential position of male and female fetuses by observing the birth of pups in unilaterally ovariectomized mothers. All of the females were injected with TP when 3 days old to induce sterility after puberty. The results showed that females located in utero between two males tended to have longer anogenital distances and to develop persistent estrus sooner than females situated between two females. The females, having resided between two males, mounted significantly more frequently in mating tests after administration of TP than females residing adjacent to females.

In addition, it is known that the male rat testis is active before birth and considerable androgen can be detected in both male and female fetuses (Resko et al., 1968; Turkelson et al., 1977). Observations such as these, together with reports that antiandrogen treatment of rats in utero results in significant blockage of masculinization (Ward and Renz, 1972), indicate that the critical period with respect to masculine development begins in the rat before birth.

The gestation period for the rat is 22 to 23 days, whereas it is only 16 days for the hamster. If the gonads were developing at roughly similar rates, then both male and female hamster fetuses would be exposed to considerably less testicular androgen than rat fetuses. Lack of androgen during the fetal period could account for subsequent diminution of tissue reactivity to androgen. Whether because of less exposure to fetal androgen or to intrinsic cellular causes, hamsters,

in general, show less responsiveness to androgen than rats do. More androgen is required to suppress permanently both ovulation and lordosis behavior in female hamsters than in rats (Swanson, 1971; Noble, 1974; Whitsett and Vandenberg, 1975). Whereas secretions from the postnatal testes diminish markedly the lordosis induced by estradiol benzoate and progesterone in adult male rats, they have much less inhibitory influence in hamsters (Swanson and Crossley, 1971). Also, it appears that more TP is required to restore sexual behavior in castrated male hamsters than in rats.

In general, data describing the degree to which normal and perinatally hormonally manipulated hamsters and rats manifest heterotypical mating behaviors can be considered as compatible with the organizational hypothesis. Also, differences between these heterotypical sexual tendencies can be encompassed, at least in part, by the hypothesis. However, whether the differing homotypical sexual responses of these same species can be explained is not clear. Are the fewer mounting responses shown by male hamsters in comparison to male rats and the longer duration of lordosis exhibited by female hamsters relative to female rats due to differences in amount or utilization of androgen during organization? While this might be an interesting and testable question, it is offered to emphasize a restriction of the organizational hypothesis. As stated, it is not intended to explain all sexual dimorphisms or species differences, or to generate experiments that can identify variables to eliminate these differences in behavior. Behavior depends not only on interrelations between the neuroendocrine system involved in reproductive pro-

cesses but also on sensory and motor capabilities and morphological characteristics of each species. At first, the hypothesis should evolve principles describing how the efficacy of activating hormones for normally occurring homotypical and heterotypical behaviors in a given species would be altered by modifying its perinatal hormone environment. In mammals, it would be anticipated that the responsiveness to hormones typically activating normally occurring female behaviors would be reduced, while those activating normally occurring male behaviors would be enhanced by perinatal androgen. Generalizing across species becomes more feasible when significant structural or morphological differences can be identified and specified as significant variables. Differences in sensorimotor capabilities, in secondary sexual organ structure, and in neural complexity could play a role in how sexual dimorphisms are expressed and, hence, how they are activated by hormones. However, facilitation or activation of sexual behavior above a reference for that species in a given circumstance should be predictable by principles common to all species.

Morphological considerations have entered into all attempts to understand the mediation of sexually dimorphic behaviors. It has been argued that the effects of perinatal hormones on behavior can be better accounted for by changes in peripheral organs than by modification of the central nervous system (Beach, 1971). Since perinatally administered TP invariably causes the clitoris to hypertrophy and assume penile characteristics, it has been argued that increases in masculine behavior displayed by treated females could be attributed to this peripheral modification. Similarly, the decrease in male sexual behavior reliably recorded in neonatally castrated males could be due to impaired phallus development. Therefore, no changes in the central nervous system have to be assumed. Considerable data have been amassed recently to render this view less plausible than when originally proposed. Androgens other than testosterone have been injected that stimulate male phallus development without major effects on the central nervous system. Neonatally castrated male rats injected with fluoxymesterone have normal penises but do not exhibit normal sexual responses (Hart, 1972).

Thus, having a normally developed, adequate penis in itself does not insure normal sexual behavior. An androgen must be present that has specific effects on the central nervous system. Another line of evidence against a simple explanation based on peripheral site of action of perinatal androgen is that male-typical responses not dependent upon distinctive peripheral organs, such as aggression, have been shown to increase in females given testosterone propionate neonatally and to decrease in males having diminished

neonatal androgen (Edwards, 1968; Bronson and Desjardins, 1970). Also, neonatal castration of male rats consistently leads to increased lordosis tendency; these males have no female secondary organs. However, despite such evidence against a complete determinative influence of peripheral structures, the expression of adequate sexual behavior clearly is partly dependent upon adequate peripheral structures. When observed, suppression of behavior must be carefully interpreted and assurances must be provided that it is not occurring because of modified or inadequate peripheral organs. It is doubtful that the peripheral-central controversy will ever be settled completely; but, at the present time, perhaps the most telling argument that perinatal hormones do not bring about their change in behavior solely by peripheral organ changes comes from current studies, such as those reported at the Work Session, directly correlating changes in neuronal structure with levels of perinatal hormones (Raisman and Field, 1973).

Attention has also been focused on the effect of perinatal hormone manipulation on the onset of puberty and on longevity of reproductive capabilities. In rats, neonatal administration of TP either has no effect or hastens the onset of puberty as measured by the date of vaginal opening (Barraclough, 1966). In hamsters, guinea pigs, and rhesus monkeys, perinatal androgen is associated with a marked delay in onset of puberty (Goy, 1970a; Brown-Grant and Sherwood, 1971). An explanation for these species differences is not available. Nor is it known whether perinatal androgen influences onset of puberty by altering ovarian secretion, timing in the brain, or somatic metabolic processes. Dose-dependent delays in the onset of puberty as measured by age of descent of testes and onset of adequate sexual behavior have been recorded in rats administered estradiol benzoate (EB) (Brown-Grant et al., 1975; Zadina, 1977). Similarly, large doses of androgen administered before the fifth day of age also delay puberty in male rats. The delay in puberty in male rats has been associated with retarded secretion of adequate amounts of follicle-stimulating hormone (FSH) (Brown-Grant et al., 1975).

A series of studies are being performed in Gerall's laboratory relating perinatal androgen treatment to longevity of estrous behavior (Gerall et al., 1979). The general finding is that perinatal androgen decreases the maximum level of lordosis responsiveness induced by dosages of activating hormone, which in most instances consists of EB and progesterone. The decrease in behavioral responsiveness is directly proportional to the amount of TP and inversely proportional to the age when administered neonatally. The rate of decline from this maximum level as a function of age is not accelerated by perinatal androgen. Longevity

of reproductive behavior is shorter in perinatally androgenized females, primarily because they have a lower capacity to utilize hormones and therefore reach a threshold of nonresponding sooner than nontreated animals. In this manner, perinatal androgen may limit the response to activating hormones for the entire life of the animal.

Attempts to find a role for ovarian secretions during perinatal development have not met with notable success. When administered exogenously, most dosages of estrogen either have no effect or decrease the capacity of the female to exhibit receptive behaviors (Gerall et al., 1972a). Evidence that perinatal ovarian secretions might have an effect on sexually dimorphic behaviors has been reported by Blizard and Deneff (1973) in rats. Also, perinatal ovarian secretion increases receptivity in neonatally androgenized female and neonatally castrated male rats (Farrell et al., 1977; Dunlap et al., 1978).

#### Sex Differences in Nonreproductive Behaviors in Rodents

Although the effects on nonreproductive behavior of manipulating gonadal hormones during the perinatal period have been much less thoroughly studied than the influence of similar manipulations on sexual behavior, it is clear that gonadal hormones can exert important organizational influences on many behaviors that are not related to reproduction in any obvious way. This fact is of such importance in assessing the relation of studies on rodents to studies of primates, including the human species, that it should be given special emphasis (see Beatty's summary of the relevant literature on this topic in table 2.2). Before presentation of the details, however, some general comments that apply to the subject of sex differences in nonreproductive behavior are worth itemizing.

1. Sexual dimorphisms in these behaviors are usually expressed as differences in the quality of behavior exhibited under specified conditions.
2. For many nonreproductive behaviors, differences in genital morphology have no obvious relationship to performance.
3. For many nonreproductive behaviors, sex differences are small in magnitude and occur as differences in average performance between groups. Sex differences in these behaviors are often markedly influenced by species, strain, and the testing conditions. Ostensibly trivial variations in experimental procedures can profoundly affect the magnitude of the average difference in performance.
4. While we are a very long way from understanding which constitutional and environmental variables are important, the available data suggesting how little we

really know about such factors should, at least, lead us to reject, as premature, attempts to explain all sex differences in nonreproductive behavior in terms of unitary and global but typically loose constructs such as "emotionality," "perceptual and cognitive restructuring," "intelligence," or "energy level."

#### Running Wheel Activity

Sex differences in running wheel activity in the rat were described more than 50 years ago when it was also shown that castration reduced activity in both sexes as well as abolished the sex difference (e.g., Hitchcock, 1925). More recent work has shown that estrogen is clearly the activating hormone in males as well as females: testosterone injections merely provide a substrate for estrogen production (Roy and Wade, 1975), perhaps in the anterior hypothalamic-preoptic area where central estradiol benzoate implants stimulate running (Colvin and Sawyer, 1969; Wade and Zucker, 1970b). Progesterone seems relatively unimportant to the average amount of wheel running except under conditions when it can inhibit the action of estrogen or substitute for corticosteroids and promote more normal metabolic function as in adrenalectomized animals. Whether or not sex differences in running wheel activity are organized by steroids during the early postnatal period is uncertain. For example, large doses of testosterone propionate (500 to 1250  $\mu$ g) within 5 days of birth reduce the response of females to activating doses of estrogen given later in life, but eventually the animals exhibit normal activity if treatment is prolonged (see Gerall, 1967; Stern and Janowiak, 1973; Gentry and Wade, 1976b). Prenatal or pre- plus post-natal treatments with androgens have not been studied.

#### Open-Field Activity

Beginning at about 50 days of age, female rats ambulate more and defecate less than males during open-field tests. Because of the long tradition in animal psychology of interpreting high defecation and low activity as indicative of high "emotionality," it has been assumed by some that female rats are less emotional than males, an assertion that has been disputed by others (see Gray, 1971, vs. Archer, 1975, for both sides of a seemingly endless debate). In contrast to wheel running, where activational effects of estrogen mainly determine activity level, in the open field activational influences of gonadal hormones on active motor behaviors (ambulation and rearing) are rather modest (Quadagno et al., 1972; Bronstein and Hirsch, 1974; Bengelloun et al., 1976; Slater and Blizard, 1976).

Castration at 30 days or later generally does not affect openfield behavior of males, but neonatal castration, especially if combined with prenatal exposure to

**Table 2.2**

Organizational and activational effects of gonadal hormones on nonreproductive behaviors in rodents [Beatty]

Behavior	Evidence for Organizational Effect Early in Development	Reference	Evidence for Activational Effects	Reference
<i>Activity</i>				
Running wheel	+	Gerall et al., 1972a; Gentry and Wade, 1976b	++	Roy and Wade, 1975 (and many others)
Open-field	++	Gray et al., 1965; Swanson, 1966, 1967; Pfaff and Zigmond, 1971; Blizard and Denef, 1973; Bengelloun et al., 1976	+	Quadagno et al., 1972; Slater and Blizard, 1976
			—	Bronstein and Hirsch, 1974; Bengelloun et al., 1976
<i>Aggression</i>				
Shock-elicited (rat)	+	Conner et al., 1969; Powell et al., 1971	+	Hutchinson et al., 1965; Bernard and Paolino, 1975
Isolation-induced (mouse)	++	Bronson and Desjardins, 1968, 1970; Edwards, 1969	++	Beeman, 1947; Edwards, 1969; Erpino and Chappelle, 1973; Luttge and Hall, 1973a,b
Spontaneous (hamster)	?	Payne and Swanson, 1972c	++	Payne and Swanson, 1971a,b, 1972a,b
<i>Sensory factors</i>				
Taste preferences	++	Valenstein et al., 1967; Wade and Zucker, 1969; Zucker, 1969; Zucker et al., 1972; Krecek, 1973; Shapiro and Goldman, 1973	+	Wade and Zucker, 1970a; Marks and Hobbs, 1972
			—	Hirsch and Bronstein, 1976
Reaction to shock	?	Beatty and Fessler, 1977a	+	Marks and Hobbs, 1972; Marks et al., 1972; Davis et al., 1976; Beatty and Beatty, 1970; Beatty and Fessler, 1977a
			—	
Feeding and body weight	++	Beatty et al., 1970; Bell and Zucker, 1971; Slob and van der Werff ten Bosch, 1975; Tarttelin et al., 1975; Dubuc, 1976; Wade, 1976	++	Zucker, 1972; Slob et al., 1973; Tarttelin and Gorski, 1973; Czaja and Goy, 1975; Gentry and Wade, 1976a; Roy and Wade, 1976; Wade, 1976
<i>Learning</i>				
Active avoidance acquisition	+	Beatty and Beatty, 1970; Scouten, 1972; Scouten et al., 1975	—	Beatty and Beatty, 1970; Scouten et al., 1975
Active avoidance extinction	?		?	Ikard et al., 1972; Telegdy and Stark, 1973; Gray, 1977
Passive avoidance	?	Bengelloun et al., 1976	—	Bengelloun et al., 1976
DRL	?	Beatty et al., 1975a	+	Beatty, 1973a
Maze learning	+	Stewart et al., 1975	?	
Taste aversion	?		+	Chambers, 1976
<i>Brain lesions</i>				
Septal area	++	Phillips and Lieblich, 1972; Phillips and Deol, 1973; Lieblich et al., 1974; Bengelloun et al., 1976	—	Phillips and Lieblich, 1972; Bengelloun et al., 1976
Caudate and globus pallidus	?		+	Studelska and Beatty, 1978
Ventromedial hypothalamus	+	Valenstein, 1968	+	Valenstein et al., 1969

++ = Sufficient data to clearly establish an effect; + = data suggestive of an effect; — = data suggest absence of an effect; ? = no data or very incomplete data.

cypoterone acetate, elevates activity to levels that approximate those seen in females (Scouten et al., 1975; Bengelloun et al., 1976). These observations suggest that exposure to androgens during the perinatal period reduces active open-field behaviors; this has also been demonstrated by studies in which female rats were injected with TP neonatally (Gray et al., 1965; Pfaff and Zigmond, 1971; Blizard and Deneff, 1973; Blizard et al., 1975).

### Aggression

There are marked species differences in the effects of gonadal hormones on aggression and differences in the testing procedures typically used with each species. Adult male mice are very aggressive toward one another until they establish social dominance relations. Females rarely fight when similarly tested. Hence, the introduction of strangers is an effective technique for inducing aggression in males, especially if the animals have been socially isolated for some time. Both activational and organizational effects of gonadal steroids control aggression in mice. Castration in adulthood abolishes aggressive behavior, and hormone replacement with androgens restores fighting in a dose-dependent fashion (Beeman, 1947; Edwards, 1969). Among androgens, testosterone and androstenedione are most effective in eliciting aggression in castrated males (Erpino and Chappelle, 1973; Luttge and Hall, 1973a), but even "strong" androgens do not elicit much aggression in similarly tested females. Aggression against a male intruder can be increased by injecting females neonatally with testosterone or estrogen (e.g., Bronson and Desjardins, 1968, 1970; Edwards, 1968, 1969; Whitsett et al., 1972).

As would be expected, neonatal castration reduces this kind of aggressiveness in male mice even if given testosterone in adulthood (Edwards, 1969). Again, the effect is time-dependent; castration at 1 day of age is more effective than castration on day 6 or later (Edwards, 1969; Peters et al., 1972). While these findings imply some sort of critical period for the organizing action of testosterone or one of its metabolites, the temporal boundaries of the critical period, if any such thing exists, are not rigidly fixed. Prolonged (20 days long) treatment with TP beginning at day 30 elevates aggression to male levels in female mice given TP injections at testing (Edwards, 1970). One mechanism by which androgens alter aggressiveness is to alter the production of urinary pheromones that stimulate attack in the mouse (e.g., Lee and Griffo, 1973).

In laboratory rats aggression is usually induced by placing pairs of animals in a small chamber and electrifying the grid with a brief but fairly intense shock. Both sexes exhibit considerable "aggression" under these conditions, but males usually fight somewhat

more than females, although the sex difference is not large. Castration reduces shock-elicited fighting in males, but the effect takes more than 3 weeks to develop (Hutchinson et al., 1965; Bernard and Paolino, 1975). Organizational effects are also present; neonatal TP in females increases aggression (Powell et al., 1971). Neonatal castration reduces such aggression in males even when they are given TP injections later in life (Conner et al., 1969). The relationship, if any, between such shock-induced aggression and more "natural" social aggressive responses is unknown.

Unlike rats and mice, female *hamsters* exhibit more aggression than males, at least in periods of diestrus. Gonadectomy reduces aggression in both males and females (Payne and Swanson, 1971a,b, 1972a). Combined treatment with estradiol and progesterone completely suppresses fighting in ovariectomized-adrenalectomized females, but neither hormone is very effective alone (Floody and Pfaff, 1977). In castrated males aggression can be increased toward intact males by ovarian transplants and TP or EB injections and toward intact females by progesterone (Payne and Swanson, 1971a, 1972b). Very little is known about organizational effects of gonadal hormones on hamster aggression. Somewhat surprisingly, neonatal testosterone treatment *increases* aggression in males to levels well above those seen in untreated males (Payne and Swanson, 1972c).

Experiments on hormonal control of aggression in male gerbils have produced highly variable results; both increases and decreases in aggression have been reported after castration or TP injections (see Saylor, 1970; Anisko et al., 1973; Christenson et al., 1973; Lumia et al., 1975; Yahr et al., 1977). Some sort of androgen-dependent, aggression-arousing pheromone also exists in this species (Yahr et al., 1977), but the confusing resulting pattern suggests that variables other than hormonal state must be important. One such factor appears to be the familiarity and neutrality of the testing arena. Gerbils also exhibit sexually dimorphic territorial marking, especially in males (Turner, 1975; Thiessen and Rice, 1976). Testosterone injections increase marking in both males and females, and the magnitude of the increase is much greater in males (Turner, 1975; Thiessen and Rice, 1976). This activational influence of testosterone reflects, in part, a direct action on the brain (Thiessen and Yahr, 1970) and, in part, peripheral changes in the ventral scent glands. Androgens also exert important organizational effects on territorial marking. Neonatal androgen treatment increases responsiveness to subsequent androgen treatment in female gerbils in an age-dependent fashion, while early castration reduces responsiveness to androgen treatment in males in an age-dependent manner (Turner, 1975).

### Taste Preferences

Female rats of several strains exhibit a greater preference than males for nutritive (glucose) and nonnutritive (saccharin) solutions (Valenstein et al., 1967; Wade and Zucker, 1969). It is important to realize that ovarian hormones are important mainly for the establishment of saccharin preference, and, once developed, the preference persists following ovariectomy. Ovariectomy greatly reduces acquisition of preference for saccharin, and only combined treatment with estrogen and progesterone restores this ability (Zucker, 1969). Activational effects of testicular hormones are relatively unimportant. Neonatal TP (but not EB) treatment, greatly reduces saccharin preference in females (Wade and Zucker, 1969). Further, feminine saccharin preferences are displayed by male pseudohermaphrodites of the Stanley-Gumbreck strain (Shapiro and Goldman, 1973); these animals are androgen-insensitive because of a genetic defect. In general, gonadal hormones affect saccharin preference in hamsters in much the same way that they do in rats (Zucker et al., 1972).

Female rats also exhibit a greater preference for salt solutions than males (Krecek et al., 1972). This difference is abolished by TP injections in 2-day-old females, but similar injections at 12 days of age are ineffective (Krecek, 1973).

### Reactivity to Shock

Female rats are more responsive to electric shock than males, as reflected in lower response thresholds (Paré, 1969; Beatty and Beatty, 1970; Marks and Hobbs, 1972) and shorter escape latencies (Beatty and Beatty, 1970; Davis et al., 1976). There is disagreement regarding the activational role of gonadal hormones in both sexes (see Beatty and Beatty, 1970; Marks and Hobbs, 1972; Marks et al., 1972; Davis et al., 1976; Beatty and Fessler, 1977a). Castration at 50 days of age lowered flinch and shuffle thresholds but left the jump threshold unaltered, while neonatal castration lowered all three of the above shock threshold measures to levels observed in females. Testosterone injections raised shock thresholds of neonatally castrated males to levels of normal males (Beatty and Fessler, 1977a); but Beatty is reluctant to interpret the effects of neonatal castration as evidence for an organizational influence of androgens because neonatal castration ostensibly renders the animal *more* sensitive to TP injections later in life.

### Feeding and Body Weight Regulation

As in many mammals, including man, the male rat eats more and weighs more than the female. A slight sex difference in body weight is apparent at birth (Slob and van der Werff ten Bosch, 1975). During the next

4 to 7 weeks of life, males generally remain somewhat heavier than females, but the difference is very small and usually does not attain statistical significance. Beginning about 40 to 50 days of age, a marked divergence in body weight begins and increases throughout life. Hormonal influences contribute to sex differences in body weight in at least three ways:

1. Ovarian hormones, specially estrogen, act to control feeding and body weight by reducing intake as long as body weight exceeds a certain "set point." Progesterone is not important to feeding and body weight regulation in females with intact adrenals, except that it may inhibit the effects of estrogen (see Wade, 1976). The onset of estrogenic regulation during ontogeny is mainly related to attainment of a minimum body weight level (Zucker, 1972). Actually what is evidently more important is the accumulation of a minimum amount of body fat (Wade, 1976). In the adult female rat, both feeding and food-motivated behavior vary with the estrous cycle; eating and weight are lowest when estrogen titers are high. In ovariectomized animals cyclic patterns of feeding can be induced with intermittent estrogen treatment (Tarttelin and Gorski, 1973). One neural target for estrogen actions on feeding is clearly the ventromedial hypothalamus (VMH). Implants of crystalline estradiol inhibit feeding in gonadectomized rats of both sexes, and estrogen implants in other parts of the hypothalamus do not suppress feeding (e.g., Wade and Zucker, 1970b). However, it is clear that estrogenic suppression of feeding and weight gain involves more than a direct effect on the VMH, since animals with VMH lesions respond to ovariectomy and estrogen injections in a way that is either nearly normal (King and Cox, 1973; Kemnitz et al., 1977) or attenuated (Beatty et al., 1975b; Nance, 1976), depending on the experimental conditions.

2. Androgens also exert activational effects on feeding and body weight gain. Castration of adult males reduces growth almost immediately and, after some delay, feeding as well. The depression of feeding is persistent, in contrast to the temporary hyperphagia following ovariectomy in the female; body weight gain is also chronically reduced. Replacement with TP increases eating and weight gain in a dose-dependent fashion; moderate doses (below 1 mg/day) stimulate feeding and weight gain. Larger doses actually depress feeding and weight gain below the level of oil-treated castrates. This effect evidently results from aromatization of testosterone to estrogen and can be antagonized by concurrent treatment with progesterone, which by itself has no effect on weight gain in the castrated male. Dihydrotestosterone has only a weak stimulating action on feeding and weight gain (Gentry and Wade, 1976a).

3. Gonadal hormones present during the perinatal period also exert important effects on the level at which body weight will ultimately be regulated. Hormone manipulations during prenatal life often reduce body weight markedly, but these changes are not easy to interpret because of the possibility of nonspecific debilitating effects. The likelihood of this possibility is enhanced because higher than normal mortality rates usually occur after such treatment. Beatty thinks this is the most reasonable interpretation of the rather frequent observation that prenatal TP treatment *depresses* body weight (e.g., Ward, 1969; Slob and van der Werff ten Bosch, 1975). Moreover, there is little or no difference in body weight between male and female rats castrated at birth; so the role of androgens in the prenatal period is probably not large. However, a single injection of TP or EB shortly after birth increases body weight levels of females in an irreversible fashion. The effect is greater in magnitude if the injections are given to gonadally intact animals, but some difference is observed in ovariectomized females, at least for TP injections (Bell and Zucker, 1971). The effectiveness of early postnatal hormonal treatment in chronically elevating the level at which body weight will be regulated is limited to a tightly demarcated period in postnatal life (Tarttelin et al., 1975), and even treatment for 20 days with large doses (2 mg/day) of TP has no effect on weight regulation when given to prepubertal females (Beatty, 1973b).

Relatively little information regarding the comparative aspects of hormonal control of feeding and body weight in rodents, other than in the rat, is available. In general, the basic effects of hormones on weight regulation seem to be similar in guinea pigs and rats, correcting for differences in the gestation period (Slob et al., 1973; Czaja and Goy, 1975), but the situation is much different in hamsters and gerbils. In the hamster gonadectomy affects neither food intake nor body weight in either sex. Progesterone elevates both measures in castrates of both sexes; testosterone depresses feeding and weight in males but not in females; while estrogen is ineffective in both sexes (Zucker et al., 1972). In the gerbil estrogen and antiestrogens (which are estrogenic in their effects on feeding (Roy and Wade, 1976)) stimulate food intake and weight gain (Roy et al., 1977).

### Learning and Performance

Perhaps because of the early discovery of estrus-linked changes in activity, there has been a long-standing tradition in animal (i.e., rodent) psychology to conduct experiments on male rats. According to Beatty, the typical justification for this widely practiced research strategy is to avoid introducing unwanted and extraneous variability that might obscure the phenomena

of interest. While this sounds reasonable enough, the strategy has been extremely costly for at least three reasons: (1) We do not possess a very clear idea of the pattern of sex differences in the many tasks that have been studied in laboratory animals over the years. (2) The hoped for reduction in variance between subjects has often not been achieved. (3) The generality of the results of animal learning experiments may be less than is typically assumed.

Despite the long tradition of designing experiments in a manner that precludes discovery of sex differences in behavior, differences in several behaviors have been described. One behavior that has been frequently studied is active avoidance, which is typically studied in one of three test situations: (1) *One way*, where one of two compartments in the apparatus is always safe and the other is potentially dangerous. (2) *Two way (shuttle)*, where both sides of a two-compartment chamber are potentially dangerous. (3) *Free operant (Sidman)*, in which shock is not signalled by a distinct external stimulus but, instead, is programmed to occur briefly every few seconds.

Female rats generally outperform males during acquisition of each of these tasks. The difference is small and rather undependable in the one-way task, probably because male rats acquire one-way avoidance quite rapidly and there really is not much room for improvement. Sidman avoidance has not been studied extensively, but a sex difference has been reported (Barrett and Ray, 1970). Most of the work on sex differences in acquisition has employed the two-way task.

In rat studies in Beatty's laboratory, organizational influences of androgens during the *prenatal* period were shown to be principally responsible for the sex difference in two-way avoidance acquisition. Activational influences of gonadal hormones seem quite unimportant, since gonadectomy has no effect on performance by either sex (Beatty and Beatty, 1970; Scouten et al., 1975). Although neonatal castration of males did not influence performance, acquisition was improved to the typically female level by combining neonatal gonadectomy with prenatal exposure to the antiandrogen, cyproterone acetate (Scouten et al., 1975). This treatment also feminized their open-field behavior (see above). While these data suggest that organizational effects of androgens on avoidance normally occur prenatally in the rat, other data demonstrate that testosterone can affect performance if given later in development. A single injection of TP at 3 days of age combined with ovariectomy and TP in adulthood caused marked impairment in the performance of females, although neither treatment alone affected avoidance behavior (Beatty and Beatty, 1970). Further, a series of postnatal testosterone injections beginning at birth and ending at 75 days of age tended to depress

( $P = 0.06$ ) acquisition by females in the tests given 2 months after injections ended (Scouten, 1972). There is an interesting parallel between the role of gonadal hormones in this sex-typical behavior and their role in sex differences in learning that is sensitive to orbital-frontal lesions in rhesus monkeys (see below).

Female rats (Denti and Epstein, 1972; Beatty et al., 1973; Bengelloun et al., 1976) and gerbils (Riddell et al., 1975) exhibit inferior performance in tests of passive avoidance behavior. As yet, no influence of gonadal hormones has been demonstrated, since castration at various ages from birth to adulthood does not affect performance in rats (Bengelloun et al., 1976).

Adult female rats acquire efficient performance on a differential reinforcement of low rates of response (DRL) schedule more rapidly than males (Beatty, 1973a; Kearly et al., 1974). At first, it appeared that activational effects of ovarian hormones were mainly responsible for the sex difference, since ovariectomy greatly impaired performance (Beatty, 1973a). However, two attempts to replicate this result have failed; so the role of ovarian hormones in this sexual dimorphism is not established (Lentz et al., 1978).<sup>2</sup> Similarly, there is no evidence that androgens contribute to the sex difference in DRL acquisition, since neither adult nor neonatal castration alters the performance of males (Beatty, 1973a; Beatty et al., 1975a). The possible influence of gonadal hormones during the prenatal period has not yet been examined.

With a few exceptions (e.g., Corey, 1930), studies that have observed sex differences in *maze learning* in rats have found that males are superior (e.g., Tryon, 1931; McNemar and Stone, 1932; Barrett and Ray, 1970; Krasnoff and Weston, 1976). The tasks that are most sensitive to the sex difference are complex mazes (e.g., the Lashley III maze) with many blind alleys. Such an apparatus is really an open field with many additional walls, and, consequently, it is not surprising that females make more errors since their greater level of activity and exploratory behavior translates rather directly into "errors" in the maze. Beatty is not aware of any systematic attempt to examine activational effects of gonadal hormones; but, if these complex mazes are really just elaborate open fields, only modest effects would be expected. Organizational effects of androgens would be anticipated, and there is one confirming report.

Stewart and colleagues (1975) reported that neonatal TP injections improved the performance of females in the Lashley III maze almost to the level of normal male controls. The same treatment also masculinized open-field behavior. There is also a report of a failure of prenatal or neonatal TP to affect Lashley III maze performance of females (Machado-Magalhaes and de Araujo-Carlino, 1974), but those workers also failed to

observe a difference in Lashley III maze performance between normal males and females. A recent experiment by Beckwith and colleagues (1977) reported a sex difference in the acquisition and reversal of a black-white discrimination in a Thompson-Bryant box. Males required fewer trials to criterion during both acquisition and reversal, and only performance of males improved as a result of neonatal treatment with melanocyte-stimulating hormone.

Recently, Chambers (1976) reported that male rats extinguish a *conditioned taste aversion* more slowly than females. Gonadectomy reduced the persistence of the aversion in males but did not affect performance by females. Testosterone treatment increased the resistance to extinction of females and castrated males. While it is possible that the phenomenon described by Chambers is a special case of altered taste preference or of extinction of passive avoidance behavior, the nature of the effects of hormonal manipulations in adulthood on taste aversion is different from the effects of similar manipulations on taste preferences or passive avoidance (cf. Zucker, 1969; Bengelloun et al., 1976). Beatty is not aware of any data on the effects of perinatal hormone manipulations on taste aversions.

#### Sex Differences in Response to Brain Damage

Goldman and colleagues (1974) reported that the effects of orbital-frontal lesions in rhesus monkeys were both sex- and age-dependent. Using three tests (object reversal, delayed response, and delayed alternation), they observed deficits in males but not in females if brain damage and testing occurred before 15 to 18 months of age. If the operations and tests occurred later in life, impairments were observed in both sexes. Since the publication of that paper, Goldman and colleagues have done additional work that demonstrates the following: (1) In young monkeys (75 days old) males perform better than females on the object reversal task, but this difference disappears as development progresses. (2) A series of postnatal TP injections (birth to 46 days of age) or prenatal TP treatment improves performance of females to about the level of young males. The number of animals in the prenatal TP group is small, but it is clear that they are not better than the postnatal despite more extensive somatic virilization. (3) Early castration does not affect male performance. The implication of these results is that androgens may affect the rate of maturation of portions of the brain that are involved in competence in performing object reversal problems but which are probably not primarily steroid targets. It will be interesting to see what happens to the performance of males exposed to antiandrogens prenatally and castrated at birth. If their performance on object reversal at 75 days is reduced, the interpretation advanced by

Goldman and colleagues would be nicely supported. In addition, there is an obvious parallel between the type of hormonal control that seems to be involved in this dimorphic behavior in the monkey and what Beatty has observed in active avoidance behavior in rats (see below).

The effects of septal lesions on emotionality in the rat are now known to be sex-, age-, and hormone-dependent. Lesions at 7 days of age and after 55 days of age result in full appearance of hyperemotionality, but similar lesions at 25 days of age are ineffective and at 30 or 45 days of age "hyperemotionality" is quite transient (Johnson, 1972; Phillips and Lieblich, 1972). Gonadectomy in males at any time other than between ages 23 to 30 days has no effect on the development of hyperemotionality when lesions are made in adulthood; between 23 to 30 days (especially 26 to 29 days of age) gonadectomy greatly attenuates the hyperemotionality from adult septal lesions (Phillips and Lieblich, 1972; Lieblich et al., 1974; Bengelloun et al., 1976). Female rats that normally show hyperemotionality even if lesions are made when they are weanlings do not show hyperemotionality if lesioned at 25 days of age if they have been given neonatal TP treatment. Conversely, neonatally castrated males show hyperemotionality from septal destruction at 25 days of age, when normal males do not exhibit the septal rage syndrome (Phillips and Deol, 1973). These results imply a complex age-dependent action of androgens in which neonatal exposure to androgen triggers some mechanism that reduces the organism's emotional reactivity following the lesion. Evidently, subsequent exposure to androgens during a remarkably tightly bounded period before puberty reverses this process, whatever it may be. The effects of septal lesions on open field, active avoidance, and passive avoidance behavior do not seem to depend on gonadal hormones, at least not in the same way as emotionality (Bengelloun et al., 1976). Unfortunately, sex differences in the effects of septal lesions on consummatory and operant behavior (Kondo and Lorens, 1971; Lorens and Kondo, 1971) have not been analyzed with regard to their dependence on gonadal hormones.

Lenard and colleagues (1975) reported that large lesions of the globus pallidus had different effects on the duration of aphagia and adipsia in male and female rats. Despite intragastric feedings, most males died without recovering voluntary feeding or drinking, but most females survived and recovered ingestive behaviors. Exactly the same pattern of sex differences in feeding occurred after intrapallidal injection of the neurotoxin, 6-hydroxydopamine (Lenard, 1977). Using smaller lesions of the pallidus, which did not cause aphagia or adipsia in either sex or affect open-field behavior, Beatty and Siders (1977) observed impaired

acquisition of two-way avoidance in both sexes, but one-way avoidance acquisition was retarded by the lesions only in males. In a parallel series of studies Studelska and Beatty (1978) observed sex-dependent effects of lesions in the ventral part of the caudate. These lesions caused transient aphagia and adipsia of comparable duration in both sexes, but impaired two-way avoidance behavior only in males. Open-field behavior and acquisition of one-way avoidance behavior were not impaired in either sex. They have also examined the influence of gonadectomy in adulthood on the effects of the ventral caudate lesions. Castration abolished the deficit in avoidance that was observed after lesions in gonadally intact males, but ovariectomy had no influence on performance in females regardless of whether or not they also had lesions. Treatment of male castrates with TP, EB, or dihydrotestosterone propionate seems to restore the effectiveness of the lesion in males, but TP injections do not produce impairments in females with ventral caudate lesions. To date, Beatty's experiments with injections of antiestrogen (MER-25 or CI 628) or an antiandrogen (cyproterone acetate) in gonadally intact males with ventral caudate lesions have not revealed any effect of these antagonists on avoidance behavior, possibly because the antiestrogens mimic the effect of EB.

The effects of both ventromedial hypothalamic and lateral hypothalamic lesions on feeding behavior and body weight regulation indicate sex differences. When the VMH is damaged by electrolytic lesions, gold thio-glucose injections, or by knife cuts lateral to the VMH region, hyperphagia and obesity are more frequently observed (or these effects are greater in magnitude) in females than in males (Valenstein et al., 1969; Wright and Turner, 1973; see also Wade, 1976; Kemnitz et al., 1977). Wade (1976) has suggested that the effect of the VMH lesion is to abolish the sex difference in food intake and in body weight gain that exists in neurologically intact males and females; i.e., after VMH damage males and females eat and gain weight at comparable rates. However, in a few studies (e.g., Valenstein et al., 1969) females gained absolutely more weight than males after VMH lesions.

Following LH lesions there is a sex difference in the level at which body weight is ultimately regulated. Both males and females exhibit aphagia and adipsia after lateral hypothalamic lesions, followed by partial recovery of ingestive behavior; but males, more reliably than females, regulate body weight at chronically lower than normal levels (Powley and Keesey, 1970). Female rats with lateral hypothalamic lesions can regulate body weight at more nearly normal levels (Harrell and Balagurs, 1975; see also Wade, 1976). These sex differences in response to hypothalamic injury are consistent with a model of sex differences in feeding recently pro-

posed by Nance and Gorski (1975). These authors note that sex differences between neurologically intact males and females on measures such as preference for strong saccharine solutions and diurnal rhythms in meal taking are qualitatively similar to the changes in feeding caused by VMH or lateral hypothalamic lesions, respectively. Thus, the feeding behavior of a normal male resembles that of an animal with a VMH lesion, while the normal female's feeding behavior is more like that of an animal with a lateral hypothalamic lesion. According to the model, neural systems that control feeding are intrinsically biased to develop in the feminine direction with an active VMH that restrains feeding and weight gain (possibly in response to changing estrogen levels; see above). Perinatal exposure to androgens might modify the development of the VMH to weaken its inhibitory control over feeding, a plausible possibility, since the VMH has been implicated as a target area for the organizational actions of androgens on neuroendocrine control mechanisms (Nadler, 1973). Moreover, neonatal treatment of female rats with TP reduces the effects of adult VMH lesions on feeding (Valenstein, 1968) and masculinizes taste preferences (e.g., Nance, 1976). However, it is not clear from the model why castration in adulthood should enhance the effectiveness of VMH lesions on feeding and weight gain in males (Kemnitz et al., 1977).

Clearly, additional work is necessary to evaluate the interactions of gonadal hormones and brain lesions that affect feeding and other behaviors. This work is needed to resolve discrepancies that have already appeared, but it is likely to be especially fruitful for another reason: brain monoamine systems that are affected by many of the lesions described above are known to be involved in many aspects of sexual and nonreproductive behavior. Recent work has shown that there are sex differences in monoamine levels in many brain regions that are influenced by organizational and activational effects of gonadal hormones (Vaccari et al., 1977; Crowley et al., 1978).

### Sexually Dimorphic Behavior in Birds

In an "ideal" bird the release of FSH by the pituitary is influenced by day length. In males FSH leads to testosterone secretion by the testes; in females, to estrogen secretion by the ovary. The presence of high levels of testosterone in males leads to male courtship. Male courtship, in turn, further stimulates FSH secretion in females so that still more estrogen is produced. Estrogen in females induces nest building, which is followed by progesterone release, leading to ovulation, incubation, and parental behavior. However, there are 8,580 living species of birds, and birds as a group show gross variability in their sexual behavior. The ideal bird is a

rather elusive creature. Some specific examples of the above-mentioned interactions between hormones, environmental factors, and avian sexual behavior are to be found in Witschi (1961), Lofts and Murton (1973), Lehrman (1964), Hinde (1970, pp. 633–640), and Hutchison (1975).

According to Nottebohm, sexually dimorphic behavior in birds falls into two rough categories that show considerable overlap: spacing behavior, which is particularly well developed in males, and reproductive behavior. When reproduction calls for spacing, the same behavior may be used to repel other males and attract females.

Spacing behaviors have the effect of apportioning resources between members of a population. The resource in question may be space or food, as when a bird stakes out a territory that includes potential nest sites as well as food resources adequate to feed itself, its mate, and its progeny. Advertising the ownership of such a large territory is usually entrusted to vocal displays, such as songs, which carry over considerable distance and penetrate dense cover. This type of breeding territory and territorial defense is common to many songbirds (Howard, 1920). In some cases spacing efforts are restricted to claiming a nest site and a minimum amount of space surrounding it. This behavior is typical of colonial breeders, such as many seabirds (Tinbergen, 1953; Nelson, 1965) or some of the weaver finches (Collias and Collias, 1967). Spacing efforts may also focus on a display arena. In this case males can be very close to each other, but defend exclusive rights to the few square or cubic yards where they advertise their reproductive availability. Display arenas have been described for grouse (Hjorth, 1970; Wiley, 1973, 1974), ruff (Hogan-Warburg, 1966), manakins (Lill, 1974, 1976), and some hummingbirds (Snow, 1968; Wiley, 1971).

Territorial displays that lead to these different types of spacing are species typical and often stereotyped, what ethologists call "fixed-action patterns" (see review in Hinde, 1970; Marler and Hamilton, 1966; Barlow, 1977). With the exception of song in some groups of birds, the motor programs responsible for the fixed-action pattern are thought to be under genetic control. The cooing behavior of doves is a good example of a fixed-action pattern used in both spacing and courtship. Cooing develops normally in young squabs reared by foster species (Lade and Thorpe, 1964) or deafened soon after hatching (Nottebohm and Nottebohm, 1971). Thus, the stereotypy and species-typical characters of these vocalizations are not learned by imitation or by reference to auditory feedback. Hybrid doves produce cooing patterns that often bear no resemblance to the rhythmic patterns of either parental species, and in extreme cases the cooing of the

hybrid is "completely disorganized" (Lade and Thorpe, 1964).

Genes may always control some parameters of sexually dimorphic avian vocal repertoires, but in some groups this control is sufficiently lax to allow for considerable amounts of learning. The song of oscine songbirds and that of some hummingbirds fall into this category, as does the vocal repertoire of parrots and their relatives (for review, see Nottebohm, 1972). In all these cases, as the bird develops its vocal repertoire, it modifies vocal output until the auditory feedback it generates matches an auditory expectation. This matching process is interrupted by deafening (Konishi, 1965; Nottebohm, 1968; review by Konishi and Nottebohm, 1969). The vocal patterns are usually so improbable or complex that it is fair to assume that for any one individual they constitute novel motor programs that would not have occurred in the absence of vocal learning.

Singing behavior is better developed among male than among female songbirds, though females of many species sing. For example, the hen of the European robin sings freely in the autumn, when male and female robins defend individual territories (Lack, 1943). Many other examples could be presented (Nottebohm, 1975), though as a group they confirm the view that, even in species where females sing, under normal conditions the incidence of such song is far below that of males. Exceptions to this are cases where both members of a pair engage in singing duets (for review, see Armstrong, 1963; Thorpe and North, 1965). It seems possible that, even in species where female song has been judged to be a rare event, it serves a purpose. For example, bow-cooing is a typical male behavior of aggressive or courting ring doves. Its occurrence is controlled by hypothalamic centers and requires testosterone (Hutchison, 1967, 1975). Yet both male and female ring doves bow-coo until they are 3 to 4 months old. Bow-cooing disappears from the female repertoire as the birds develop into breeding condition, which occurs at 5 to 6 months (Nottebohm and Nottebohm, 1971). Whether bow-cooing in young female ring doves is a meaningful reflection of the developing endocrine system or an important step for normal socialization is not known.

We can assume that female oscine songbirds, which normally sing, develop their song as a vocal learning process, as the males do. An intriguing example is the sexually dimorphic vocal repertoire of the Indian hill mynah. In this species males in a particular area imitate calls of other males in that area, whereas females imitate only female calls (Bertram, 1970). Such sex-restricted imitation depends on recognition of the sex of the potential model, not a simple task in a species lacking morphological sexual dimorphism. In some

species, such as the South American rufous-collared sparrow, female song may occur rarely and then only early in the breeding season. Nottebohm pointed out that two female sparrows that he has collected produced songs that were close replicas of the dialect characteristic of the local population. The dialect is known to be a learned trait for the local population; therefore, by inference, the two females had also learned it.

Nest building is another complex sexually dimorphic trait. The extent of male or female participation varies among species. Both sexes of ring doves cooperate in nest construction, the male usually gathering material and carrying it to the female, who stands at the nest site and constructs the nest (Lehrman, 1964). It is the female canary that constructs the nest (e.g., Hinde and Steel, 1966), but the male European wren constructs a number of nests. After the male forms a pair bond with a female, she will select one of the nests and provide the soft lining (Armstrong, 1955). Multiple nest building is also shown by male long-billed marsh wrens (Verner and Engelsens, 1970) and by male weaverbirds. This latter African finch weaves a domed nest out of grass or palm leaf strips (Collias and Collias, 1962), and the quality of the nest improves with practice (Collias and Collias, 1973).

Song learning and nest building are both under hormonal control and experience can play a role in both. However, they may differ in that song learning leads to the acquisition of new motor patterns, whereas improvements in nest building may result merely from better selection and handling of building materials.

There is at least one case where the genetic contribution to nest building has been demonstrated. Some African lovebirds of the genus *Agapornis* carry nesting material in their beak. Other lovebirds of the same genus, but different species, carry nesting material by sticking it under the feathers of their rump. Although hybrids try both patterns of carrying nesting material, they never succeed in rump transportation. With practice these more ineffectual attempts are abandoned (Dilger, 1962).

Vocal displays and nest building were emphasized above because they are sexually dimorphic behaviors particularly well developed in birds; but other sexual behaviors are still to be considered. With the exception of copulation and egg laying, the performer's sex in other reproductive roles varies considerably among species and groups of birds. In this sense birds show a remarkable diversity of evolutionary adaptations. For example, something as basic as incubation can be the sole responsibility of females, as in the case of fowl, ducks, hummingbirds, manakins, and most oscine songbirds where the female has a dull coloration. In doves and some parrots both sexes incubate, though the female still does more and is responsible for the

longer night shift. In some shorebirds the decision of who incubates what can be a complicated one: in the spotted sandpiper, males incubate the first clutches and polyandrous females share in the incubation of the final clutch (Hays, 1972). In the sanderling, two clutches are laid in separate nests and the pair bond dissolves before incubation begins. Each member of the pair incubates one of the clutches and attends the ensuing young (Parmelee, 1970; Parmelee and Payne, 1973). In some groups of birds females have liberated themselves of all maternal chores other than egg laying: in rheas, tinamous, jacanas (Jenni and Collier, 1972), and phalaropes (Höhn, 1967), it is the male that does all the incubating. Interestingly, in species such as domestic fowl, mallard, and red-winged blackbird, where the female has retained sole responsibility for incubation and care of the young, there is a higher content of testosterone in the testis than in the ovaries; in pigeons, where both sexes share in incubation and care of the young, and in the phalarope, where these roles are the sole responsibility of males, testosterone content (in micrograms per gram of tissue) is higher in the ovaries than in the testis (Höhn and Cheng, 1967). Interpretation of this suggested relationship is difficult, since the relation between ovarian testosterone content and follicular events has not been worked out. The higher ovarian than testicular concentration of testosterone may be a transitory and not a persisting characteristic.

In some truly modern species, neither sex builds a nest nor incubates. These are the brood parasites, such as the New World's cowbirds (genus *Molothrus* (Rothstein, 1975; Friedmann et al., 1977)), Africa's parasitic indigo birds (genus *Vidua* (Nicolai, 1964; Payne, 1973)), and the South American black-headed duck (Weller, 1967). But perhaps the most remarkable arrangement of all is that found among the megapodes of the South Pacific. In this group of galliformes, incubation heat is solely provided by environmental sources. Brush turkeys, for example, construct mounds of plant material and the male regularly tests the temperature of the mound by probing with its bill. In the first burst of fermentation, the temperature of mounds rises to a high level and the male digs into the top, turning and mixing the material. Not until the temperature is declining does he permit the female to approach and lay eggs. Throughout the incubation period the male remains in charge of the mound and exercises some control over its temperature (Frith, 1964).

Nest building, copulation, incubation, and parental duties are usually preceded by courtship. Evolutionary forces have used every possible stratagem to create the most varied courtship patterns. Australian bowerbirds have evolved polychrome nuptial palaces decorated

with flowers, berries, and shells (Gilliard, 1969). The Australian lyrebird has evolved a baroque and exquisite song dance. Such hypertrophied male displays seem to occur in species with very brief sexual encounters. While the males are polygamous, all postcopulatory reproductive roles are left to the females. In the more common situation of monogamous species cementing the bond between the sexes, all steps leading to copulation are slower and require some degree of intimacy. Well-worked examples are the displays of grebes (Huxley, 1914), gulls (Tinbergen, 1953), ducks (Lorenz, 1941), and some songbirds (e.g., Marler, 1956). Many of these behaviors are ritualized (e.g., Morris, 1957) and lend themselves well as visual signals conveying the reproductive intentions of both partners.

Courtship displays convey sexual and species-specific information. Their most obvious role is to ensure that sexual interactions occur between members of the same species and opposite sex. Courtship is also thought to *synchronize* the behavioral rhythms of prospective partners, *trigger* adequate behavioral responses, and *direct* these responses so that the interacting individuals are properly oriented (Tinbergen, 1951; Lehrman, 1964).

Courtship displays often include two or three interacting motivations, subsumed as aggression, fear, and sexual drive (Tinbergen, 1952, 1954; Hinde, 1953; Moynihan, 1955; Morris, 1957). Successive displays are supposed to allay the element of fear, reduce aggression, and permit the full expression of the sexual drive. This theory is supported by the fact that many courtship displays include components or modified parts otherwise known to occur as part of pure threat or pure escape behaviors. The overlap in courtship between sexual and agonistic tendencies is understandable if we think that the close proximity required by copulation necessitates a degree of boldness by the sex initiating the encounter. This boldness, or aggression, is otherwise encountered in dominance relations, for example, in wintering flocks, where aggression secures access to a scarce resource such as food. Those experienced with the displays of doves and domestic fowl must have pondered whether all male courtship has not evolved from these more primitive, highly aggressive approaches. It is interesting that in some primates we see the reverse borrowing of display elements; in this case sexual displays are used in agonistic situations so that, for example, presenting and mounting are, respectively, extreme forms of submissiveness and dominance, and in this context they can be shown by either sex (Wickler, 1967).

The dependence of avian sexual displays on an adequate complement of hormones has been forcefully demonstrated by the work of Lehrman and associates

at Rutgers, and by Hinde and collaborators at Cambridge (Lehrman, 1964; Cheng and Lehrman, 1975; Hinde, 1965, 1970, p. 636). The work of these authors has also shown how external stimuli, such as day length, access to nesting material, and occurrence of sexual displays, affect levels of circulating hormones; and how the opportunity to perform certain behaviors, in turn, brings about further hormonal changes. From this viewpoint the courting interactions between members of a pair can be seen as a way of bringing into step the physiology of both partners in preparation for the subsequent parental duties of incubation and care of the young. It should be noted, though, that in birds as in mammals, despite the information content of sexual displays and despite the hormonal control of these displays, "mistakes" can occur, leading to homosexual behavior in male-male and female-female pairs (e.g., Buchanan, 1966; Hunt and Hunt, 1977).

There is one other potential role of sexual displays that has not yet received proper attention: the role as *fitness predictors*. Each sex runs the risk of selecting as a mate an inferior individual. In polygamous species with display arenas, only a few males succeed in attracting females. These successful birds are older ones and hold central territories obtained after months, sometimes years, of fierce competition (Wiley, 1973, 1974; Lill, 1976). Their chance of leaving progeny is maximized by inseminating all comers. Females minimize their risk of choosing an inferior partner by selecting these central "winners." In the more common situation of monogamous species, each individual, regardless of sex, should strive to find an optimal partner. Traits sought after should be endurance to survive environmental extremes; aggressiveness in competing for mates, territories, and food; a physiology honed for optimal food utilization; disease resistance, etc. Each sex should seek a partner that will excel in the fulfillment of parental duties, that will show zeal in incubation and efficiency in feeding and defending its young. Much as in the old times, the father of the bride demanded a financial report from his aspiring son-in-law, the male and female of a prospective pair should demand from each other a fitness report. As courtship displays evolve, each sex should seek in its counterpart behaviors that are good predictors of genetic fitness and future parental performance, and should respond favorably to those behaviors. The influence of nutrition on reproduction has been described in several mammals (Sadleir, 1969). Recent studies suggest that undernutrition affects the levels of gonadotropic hormone-releasing hormones, which in turn affects testosterone secretion (Millar and Fairall, 1976). In chicken and white-crowned sparrows, restricted access to food results in reduced testicular size (Parker and Arscott, 1964; Miller, 1970), and, presumably, such

testis secretes less testosterone. Success at finding food is surely one component of fitness. Behaviors dependent on testosterone levels, such as aggression and birdsong, could give a good quantitative report on this aspect of the fitness of an individual; thus, a female may be well advised in responding selectively to such displays. Nottebohm has less of a feeling for what may be a fitness predictor in females. In the golden-headed manakin females visiting a display arena respond to a courting male by performing some of the acrobatics used by displaying males (Lill, 1976, p. 12). In the chaffinch the female's initial response is submissive, yet becomes more aggressive before copulation (Marler, 1956). In gannets females approach males in a submissive manner and males receive them aggressively, biting them (Nelson, 1965, pp. 266–268). This agonistic nature of the bonding ceremony is so marked that Nelson remarks that male aggression and female tolerance must have evolved in linkage. We may wonder whether male aggression and female tolerance to aggression may not be a measure of the same underlying factor, an element of toughness or "courage." Much as it takes courage to attack, it also takes courage not to turn and flee. Are female displays controlled by a molecule or molecules whose circulating levels, as suggested for male testosterone, bear a relation to long-term health and reproductive fitness? The choice of partner should be equally meticulous by male and female. Male displays tend to be vigorous, boisterous: a surplus of nests, cascades of song, strutting, parading, and aggression. If these are, in part at least, ways of displaying fitness, we can only conclude that females are more subtle in putting forth their case!

Why is it that a particular hormone, or mix of hormones, has come to influence the ontogeny and manifestation of various reproductive displays? Why is it that a particular set of courtship behaviors has become necessary for pair formation and necessary to achieve the synchrony required by sexual reproduction? Part of the answer, at least, may be that each sex demands a credible fitness report from its future partner.

### **Sexual Dimorphisms in Nonhuman Primates**

While perhaps not so numerous as avian species, the 11 families and 60 genera of primates present an astonishing array of adaptations in morphology and behavior. In size alone, they vary from the pygmy marmoset and mouse lemur (*Microcebus*), which average only about 70 g in adult weight, to the huge gorilla, which may attain an adult body weight (in males) of 275 kg. Not less varied are their social adaptations, which range from the virtually semisolitary life-style of the orangutan to those complex, highly structured societies (some containing more than 200 individuals) char-

acteristic of some monkeys and baboons. A number of recent books summarize the social adaptations of non-human primates, especially those from the Old World, whose evolutionary biology more closely parallels that of human beings (Southwick, 1963; Kummer, 1971; Rowell, 1972; Lancaster, 1975). These summaries are based on data gathered during field studies, and, accordingly, the results are primarily descriptive and do not represent the level of analysis of causation that normally characterizes experimental work. Similarly, the observations have been carried out by different workers having different viewpoints and interested in different problems. These differences, however, do not in any way account for the recorded differences in social adaptations any more than they are able to account for reported concordances and similarities. Thus, the existence of monogamous pair bonds that may last throughout adult life is an established fact for species as distantly related as the gibbon ape of Asia and some of the marmosets of South America. The occurrence of monogamous mating in these primates is associated with an absence of sexual dimorphism in body size, and, perhaps to a lesser extent, with an absence of sexual dimorphism in behavior (Carpenter, 1942). For the marmoset, even parental care tends to be equally provided by both parents except for the restriction of lactation to the female (Eisenberg, 1972). For nonhuman primates, in general, it can be said that a strong relationship exists between sexual dimorphism in body size and the system of mating that is characteristic of a species (Leutenegger, 1978).

Variations in social adaptations have encouraged field workers to develop a primitive classificatory system delineating the basic social unit for each species. Most often, this basic social unit has two features that differ conspicuously among different populations. They are (1) the socionomic index (the ratio of adult females to adult males), and (2) the system of mating. These two features are independent. Thus, when the socionomic index is 1.00, the system of mating may be monogamy with permanent or enduring pair bonding, and the basic social unit is the bonded pair and their offspring, or it may be complete promiscuity with transient pair bonding during the consortship. Generally among Old World terrestrial monkeys and baboons, the socionomic index is greater than 1.00, sometimes approaching 4.00 in rhesus (Lindburg, 1971); but the system of mating can be either promiscuous or an entirely different sort, such as the harem type.

Although an association clearly exists between habitat and the basic social unit of nonhuman primate groups, this association represents a remote evolutionary causation (i.e., selection for a particular adaptation), and contemporary changes in habitat are often

without effect in modifying the species-specific social unit. Kummer (1971), for example, describes specific social organizations for hamadryas and anubis baboons despite overlapping geographic habitats. Hamadryas baboons typically have evolved a three-level society consisting of (1) the troop, (2) the medium-sized band, and (3) the one-male unit, which consists of a single male and his permanently bonded adult females and their dependent offspring. Anubis baboons, in contrast, have evolved a multimale troop consisting of a small group of males living continuously with a large number of females and their dependent offspring. In this latter social organization the mating system is accurately described as promiscuous, and only brief and temporary bonds occur between mating individuals. In the former case (the hamadryas), mating, though polygynous, is restricted to the one-male unit, and matings with females outside this unit have not been reported even for females belonging to the same band or troop. In their natural habitats, anubis and hamadryas also differ in their sleeping habits, the former roosting in trees and the latter lodging on cliffs.

As Kummer (1971) reports, anubis and hamadryas occupy adjacent niches in the Awash National Park in Ethiopia. At the boundary of their niches, hybrids are found that have a curious mixture of hamadryas and anubis characteristics in their social organization. Where there are one-male groups, these are small and unstable, and the successes of males in forming harems are meager. Within this park, the ecological transition from forest to cliff country corresponded exactly with changes in sleeping habits for both species, such that anubis now slept in cliffs and hamadryas in trees, but social organization was not similarly affected by the ecological change. "Anubis groups without one-male groups, hamadryas troops with one-male groups, and the in-between societies of the hybrids all occurred in the same canyon habitat" (Kummer, 1971, p. 135). Thus, while some behavioral traits are clearly flexible and adaptable to changing habitats, others (like social organization) are more rigid and more constantly associated with genotype than with habitat.

The tendency to pair bonding and monogamous mating is so strong among marmosets that it can be manifest even in the laboratory environment when a female is housed simultaneously with two males (Epplé, 1972). Similar dispositional tendencies have been noted for the monogamous New World titi monkey (Mason, 1978). Thus, the tendency for the bonded male and female with their dependent offspring to constitute the basic unit may be genetically rather than environmentally determined, and it is conceivable that the multimale troop might be genetically based as well.

Efforts to characterize the structure of nonhuman primate societies at levels more analytic than mere

description of the ratio of males to females and the systems of mating involved have relied on two different, but not necessarily mutually exclusive, concepts: (1) the dominance hierarchy and (2) social roles. Within the context of sexually dimorphic behaviors, both concepts have relevance.

Dominance is most often defined in terms of performance measures. The defining behaviors are either the frequency of aggressive behaviors, the frequency of submissive behaviors, or the number of troop members toward which either of these behaviors is displayed. Less often dominance is determined by dyadic competition for a valued incentive. For example, the observer may toss an apple approximately midway between two monkeys and record which obtains possession. Obviously, use of the term dominance should not go beyond its defining operations, but it nearly always does so. To say that animal A is dominant to animal B merely summarizes a behavioral relationship between them, and it neither "explains" nor provides insight into the reasons for that relationship. Nevertheless, many workers use the term dominance as though it explains why animal A aggresses animal B and the reverse never or only rarely occurs. Despite its shortcomings and misuses, most Old World terrestrial monkeys and baboons display hierarchical organization of their aggressive-submissive interactions. One or a few of the animals are never threatened or aggressed by the others, and, at the opposite end of the hierarchy, one or a few animals are threatened or aggressed by nearly everyone on occasions of dyadic interaction that are appropriate to the elicitation of such behaviors. Quite regularly among these species, one or a few males are at the top of this hierarchy. This is a regularity that cannot be accounted for by physical size, since, clearly, if that were the only determinant all adult males in the society would be distributed among the top ranks, and this is not what occurs. For our purposes, however, it is important only to note that the top ranks are consistently associated in some species with the male sex.

In nonhuman primate societies for which dominance hierarchies are inappropriate, or as an alternative to that notion, investigators have developed and utilized the concept of social role. First suggested for rhesus monkeys by Bernstein and Sharpe (1966), the concept has also been useful for the baboon (Rowell, 1966), the African vervet (Gartlan, 1968), and Japanese macaques (Eaton, 1976). Social roles for members of these societies are, in part, related to age and dominance rank, but even more strikingly to sex. Thus, Eaton (1976) describes a role specific to the alpha male Japanese macaque (directing movement of the troop). In addition, subleader males assist the alpha male in "policing" and defending the troop against predators. Adult females, in contrast, do not take part in these

activities to any great extent, and their role is described primarily as raising and protecting infants and defending female allies in intra-troop aggressive encounters. For vervet monkeys, Gartlan (1968) describes interfering in intra-group aggressing as exclusively an alpha male role, and the chasing of intruders out of the group territory as a role for the alpha and juvenile males. In his study, females were more often the initiators and receivers of friendly approaches than males of any age class. Gartlan emphasizes that learning plays a significant part in the assumption of social roles. Although biological factors may determine the range of social roles an animal performs, "there is no evidence that particular roles are associated with different [generically determined] levels of biological fitness ..." (Gartlan, 1968, p. 115).

It should never be assumed from what has been said that all Old World primate groups are organized in such a way that an alpha male can always be identified, or that the role of the alpha male is the same from one species to another. For example, the *patas* monkey is an Old World terrestrial species generally found in one-male units or groups. But the females in the band lead the movements of the group and the single male does not play the kind of leadership role characteristic of the hamadryas male with his harem (Kummer, 1971). Moreover, females in the *patas* band seem quite able to control which male will remain with the band through the mechanism of initiating copulation only with the preferred male and aggression directed toward the nonpreferred male (Gutstein, 1978). Under the social system of the *patas*, the single male in the one-male band may not be the alpha animal at all, and social roles conceptualized as stereotypes of masculinity may be the prerogatives of females (Rowell, 1978).

In general, studies of feral primates have paid less attention to social roles of infants and juveniles than to those of adults. While most workers would agree that primates as a class are unique in their prolongation of the period of extrauterine dependency of offspring, only generalizations concerning its significance have been offered. Thus, Lancaster (1975) points out that the freedom of offspring from the responsibilities of adult social roles provides a protracted opportunity to learn complex social skills essential for later roles. The primary vehicle for learning such skills is postulated to be play, and juveniles of most primate species display high levels of social play. In this context, if the division of adult social roles according to sex that characterizes some primates is considered, it is not too surprising that forms of juvenile play also differ according to sex. Males show rougher and more vigorous forms of play than females, and females show more interest in individuals younger than themselves than males do. The former of these juvenile patterns, namely the disposi-

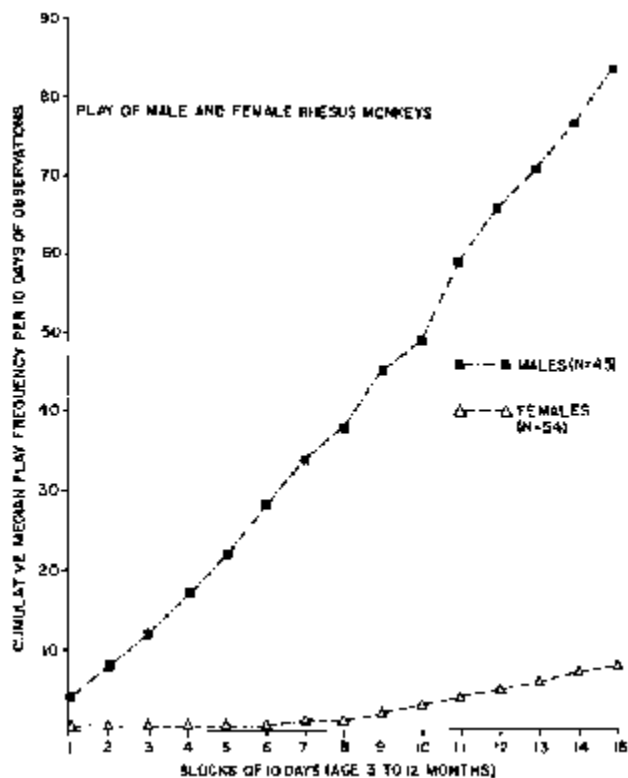


Figure 2.1

Sex differences in the frequency of performance of rough play by rhesus monkeys during the first year of life. [Goy]

tion of males to show rougher and more vigorous play than females, has been studied in the laboratory as a sexually differentiated trait in rhesus monkeys (Goy and Phoenix, 1971). In that study, males and females taken from their mother at 3 months of age were shown to display differentiated patterns of development of rough-and-tumble play. Males performed this behavior about twenty times more frequently than females. More recently, the development of this behavior has been studied in a situation in which mothers and infants of both sexes were continuously present throughout the first year of life. The presence of mothers throughout this period of development neither diminished nor enhanced the sex difference in performance (figure 2.1), and males continued to outperform females. In another study aimed at determining whether the presence of males actively inhibited performance by juvenile females, groups containing only mothers and their female offspring were studied (Goldfoot and Wallen, 1978). The frequencies of performance of rough-and-tumble play by juvenile females reared with only females present were not different from those shown by females reared in heterosexual groups. This finding is in essential agreement with one previously reported (Goy, 1968) that utilized a mother-free testing situation. Thus, the tendency of female rhesus

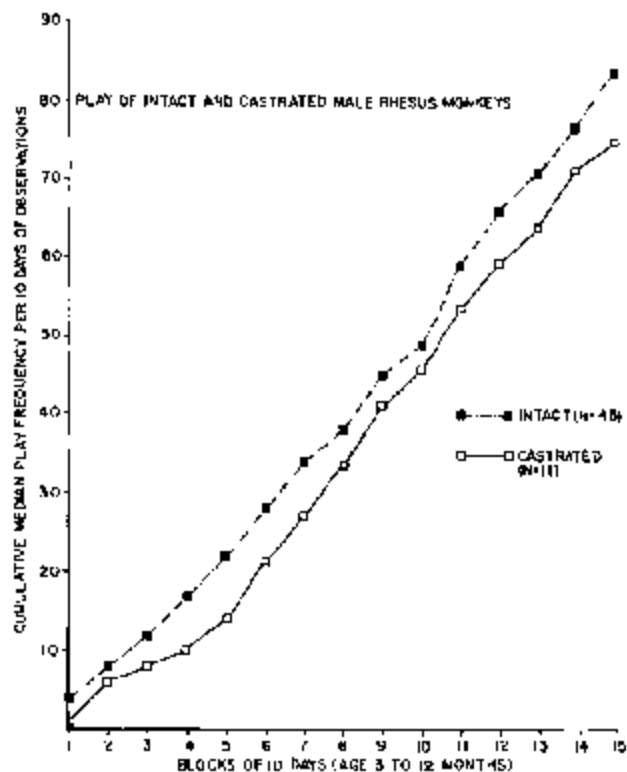


Figure 2.2

Lack of effect of castration during the first 3 months of postnatal life on the performance of rough play by male rhesus monkeys. [Goy]

us monkeys *not* to engage frequently in rough play is not easily modified by environmental influences.

The tendency of males to display high frequencies of rough play is not related to secretions of the testis after birth. When male rhesus monkeys were castrated at any time from the day of birth to 3 months of age, their subsequent display of rough play equalled that of normal intact males (figure 2.2). Moreover, rough play behavior is not a male trait that depends directly on the presence of a Y-chromosome. Rather, high frequencies of performance of this behavior depend indirectly on the Y-chromosome; i.e., on the secretory products (the androgens) produced by the Y-determined gonad. When genotypic rhesus females are exposed to suitable androgens during the appropriate period of prenatal development, they show rough play postnatally in higher frequencies than normal females (figure 2.3). Postnatal treatments of genetic females with potent androgens are ineffective (Joslyn, 1973).

The possibility that the juvenile ovary might actively suppress rough play in normal females has also been studied (Goy, 1970a). It does not, and females ovariectomized on the day of birth show levels of rough play that are not different from those of normal females. Moreover, prenatally androgenized female rhesus monkeys display heightened levels of rough play compared

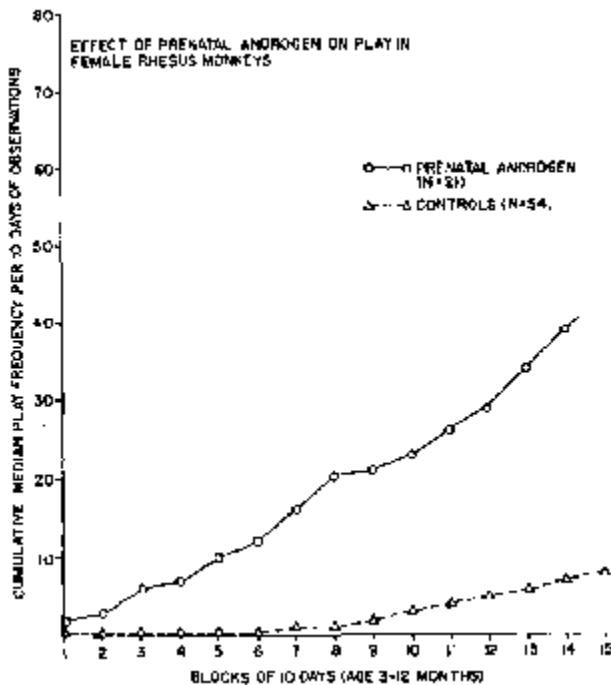


Figure 2.3

Effect of prenatal androgen on the frequency of performance of rough play by rhesus females during the first year of postnatal life. [Goy]

to normal females, although there is nothing discernibly different about their prepubertal ovaries compared with normal females' ovaries.<sup>3</sup>

Mounting behavior has also been shown to be sexually dimorphic in laboratory studies of juvenile rhesus monkeys (figure 2.4). Like rough play, its frequency of performance is unaffected by neonatal castration (figure 2.5), and genetic females can be induced to mount significantly more often than controls if they are exposed to androgens prior to birth (figure 2.6).

During the juvenile period, mounting behavior is clearly unrelated to reproduction; partners of both sexes are mounted frequently, and the function(s) it might serve is(are) not known. It seems clearly to be a part of the gestural and expressive repertoire, and, as such, some learning of when to mount, whom to mount, and where to mount may be required. The performance of this behavior is very much influenced by social history and testing conditions (Goy and Wallen, 1979). Moreover, the frequency of performance of this behavior can be increased in untreated females by testing them in isosexual (all female) groups (Goldfoot and Wallen, 1978). Isosexually tested females, however, do not become as proficient mounters as control males or prenatally androgenized females.

The reason why females mount only infrequently in heterosexual groups is obscure, but laboratory findings on this point are consistent with those from field

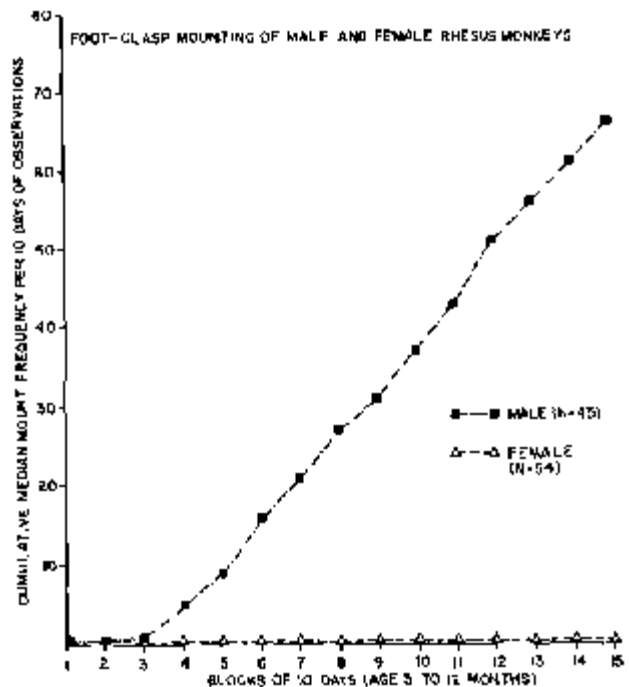


Figure 2.4

Sex differences in foot-clasp mounting behavior in rhesus monkeys during the first year of life. Note that data are plotted as medians, and the medians for females were never greater than zero despite frequent mounting by a few individual females. [Goy]

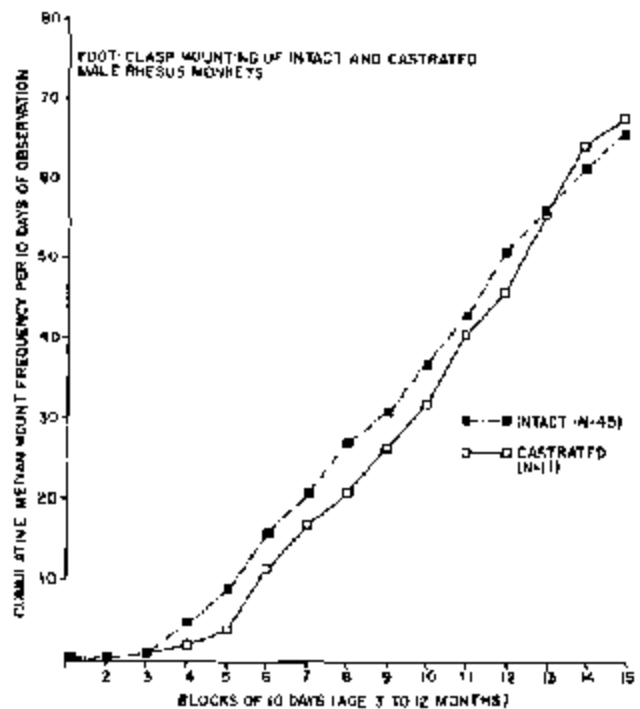


Figure 2.5

Lack of effect of castration during the first 3 months of postnatal life on the frequency of performance of foot-clasp mounting by male rhesus. [Goy]

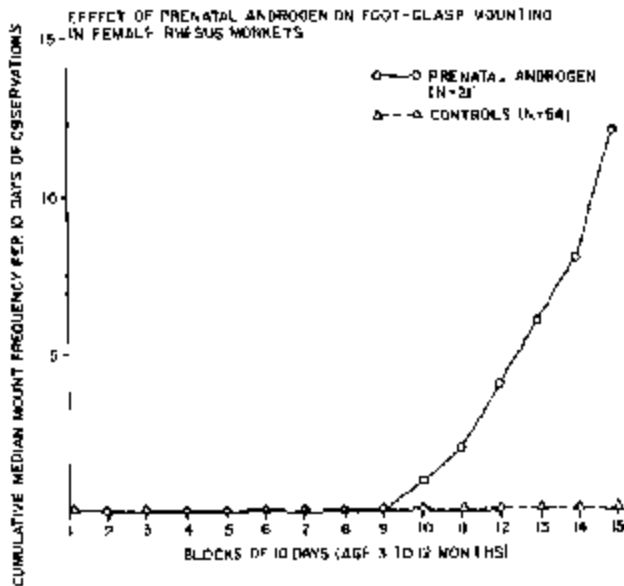


Figure 2.6

Effect of prenatal androgen on the frequency of performance of foot-clasp mounting by rhesus females during the first year of postnatal life. [Goy]

studies (Lindburg, 1971). Perhaps the reason is that mounting partly defines the role of more dominant members of the juvenile group. In heterosexual groups of juveniles, the status positions in the upper end of the dominance hierarchy are nearly always filled by male (or androgenized female) members of the group. In isosexual groups, in contrast, only females can be in these higher positions.

Studies of dominance, based on submissive interactions (Goy and Goldfoot, 1974), have been completed recently on six heterosexual groups of rhesus weaned from their mothers at 1 year of age. Five of these groups contained three males and two females and one contained four males and two females. Thus, the groups contained 31 subjects including 19 males and 12 females. Males were ranked alpha in five, and a female was ranked alpha in only one of these six groups. Thus, males achieved alpha more often than chance expectancy (3.67) and females less often than chance expectancy (2.33).

In 18 heterosexual groups of juveniles (studies starting after weaning at 1 year of age) that contained prenatally androgenized females as well as normal subjects, the alpha status was held by males nine times, by prenatally androgenized females six times, and by control females three times. These empirical determinations compare with corresponding chance expectancies of 6.78 alpha positions for males, 4.51 for prenatally androgenized females, and 6.70 for control females. In summary, in these groups males and prenatally androgenized females held alpha positions more often than

expected by chance, and control females held such positions less often. Thus, in laboratory groups that in few respects resemble natural social groups, the tendency of males to hold the higher dominance positions is as characteristic as it is for feral troops. Moreover, prenatal androgenization of the genotypic female produces an individual that is indistinguishable from a normal male in its disposition to high dominance status even when it is studied in a group that contains normal males as peers.

### Biological and Environmental Determinants of Sex Differences in Behavior of Humans

Ehrhardt stated at the outset that there are few behaviors in humans that meet the criteria for being called sexually dimorphic, and that among those behaviors where sex differences do exist there is often considerable overlap of the trait distributions for the sexes. Moreover, she stated that the impact of social factors and the environment is presumed to be much greater in humans than even in nonhuman primates. Nevertheless, she believes that it is fallacious to attribute entirely to environmental factors all those sex differences that can be measured.

Ehrhardt lists the following categories of sex differences in human behavior: (1) *sexually dimorphic behaviors* (e.g., maternal behavior) for which one can find somewhat comparable animal models; (2) *cognitive sex differences* (e.g., verbal and spatial abilities) that may have measurable counterparts in lower animals; (3) *gender identity* (i.e., self-identification as male or female, which carries with it the implication of self-awareness) for which there may be no measurable counterpart in other species; (4) *gender role behavior* (e.g., the classification of different games or sports into boys' and girls' activities, or corresponding classifications of adult careers or occupations, e.g., "housewife"), (5) *sexual orientation*, i.e., the choice of a sexual partner (homosexual, bisexual, or heterosexual), for which there is some disagreement among various experts on the existence of animal models.

Whereas it is manifestly impossible, in her opinion, to equate animal behavior with certain human responses (e.g., lordosis in a male rat in response to another male and a man falling in love with another man), it is possible to make some more abstract analogies between behaviors seen in lower animals and in humans and to evaluate effectively biological determinants of some selected human behaviors.

The approach that Ehrhardt, Money, and co-workers have pioneered is based on the measurement of gender identity and a related but independent parameter, gender role behavior, in individuals suffering from congenital or drug-induced abnormalities of

**Table 2.3**  
Human sexual differentiation [Ehrhardt]

Syndrome	Genotype	Gender Role Behavior	Reference
Turner's syndrome	XO	Feminized	Ehrhardt et al., 1970
Androgen insensitivity	XY	Feminized	Money et al., 1968 Masica et al., 1971
Adrenogenital syndrome	XX	Masculinized	Ehrhardt, 1973, 1977b

body sex differentiation; for example: the androgen-insensitivity syndromes ([...]); *Turner's syndrome* (absence of one sex chromosome and deficiency of gonadal tissue); the *adrenogenital syndrome* (congenital adrenal hyperplasia, *CAH*, or the variety induced by androgenic drugs, such as Provera, given to counteract toxemia in pregnancy) in which there has been some masculinization of reproductive tract, external genitalia, and body type. These conditions are summarized in table 2.3, together with the general trend of gender role behavior in such individuals, which is seen to be consonant with endocrine status rather than genetic sex.

Ehrhardt elaborated on this, using as an example the adrenogenital syndrome (*CAH*). In recent studies, she and Baker (1974) compared *CAH* females (who were reared as females after surgical correction) with female siblings. These individuals showed higher activity levels than their siblings, tomboyishness, preference for male friends, and less interest in dolls.

These findings of masculinized gender role behavior in *CAH* females, in spite of their being reared as females, are particularly striking. Ehrhardt stated that they could find no consistent factors in the mothers' behavior toward affected and nonaffected daughters. Likewise there were no significant birth-order differences between the two groups. Among the *CAH* females there was a tendency for the most strongly virilized individuals to be the most active and tomboyish. While the exact forms of play that characterize prepubertal male humans and prepubertal male rhesus may not be identical, both of these primates show a clear division of preadolescent activities into gender roles. In both species males show more active and rougher forms of play than females, and the behaviors characteristic of each role may serve comparable functions and may also represent pure homologies. In both species, moreover, females androgenized prenatally assume the male gender role and display its associated behaviors prepubertally. Ehrhardt was then asked whether the higher activity level in *CAH* females (and also in normal males) might be the primary factor in

differences in gender role behavior whereas the other traits, such as preference for male friendships, might be secondary manifestations. She replied that this might be so, but that the fantasies of these children, as manifested in altered patterns of doll playing and rehearsal for marriage and motherhood, could not be accounted for in this manner. Asked about sexual behavior of *CAH*, she replied that these *CAH* females were all heterosexual, except for one bisexual individual. A parallel study by Money and Schwartz (1978), with similar observations, led to the conclusion that altered prenatal environment in no way dictates postnatal sexual orientation even though the androgenizing prenatal condition can profoundly alter prepubertal gender role behavior.

An earlier study by Ehrhardt and Money (1967) found that females masculinized in utero by exposure to androgenic progestins showed, as in *CAH* females, elevated activity levels, preference for male friends, tomboyishness, interest in a career over motherhood, and decreased interest in appearance and doll play.

Besides the effects of adrenal androgens or androgenic progestins in the direction of masculinizing gender role behavior, there are also reports of estrogens and progesterone having the opposite effects, namely, feminization or inhibition of masculinization. Yalom and colleagues (1913) found that males of diabetic mothers exposed to elevated exogenous estrogen prenatally showed lowered aggressiveness and assertiveness and less athletic skill and other kinds of masculine interests, as well as retarded heterosexual development. The finding of estrogenic "feminizing" effects finds some parallels in studies on feminization of male rats by estrogens given shortly after birth (see above) and is not inconsistent with the organizational model described earlier. Estrogens may be acting to inhibit gonadotropin secretion and to interfere with testicular development during differentiation. Results obtained with estrogens are difficult to interpret, however, because these hormones are known to act differently in different species and in some species may have paradoxically masculinizing actions.

In their study of female and male children, aged 16 to 19, of mothers given progesterone (not the androgenic synthetic progestins referred to above) for toxemia in pregnancy, Zussman and colleagues (1975) found lowered physical activity and tomboyishness, and elevated interest in appearance. Ehrhardt and colleagues (1977) studied a comparable situation, girls exposed in utero to medroxy-progesterone acetate vs. matched controls. Though younger (8 to 12 years old) than the children in the Zussman study, these subjects showed a significantly greater preference for feminine clothing styles and a tendency for showing less verbal and indirect aggression, as well as a nonsignificant ten-

dency for lower energy levels, lesser athletic skills, and lesser tomboyishness. It would thus appear that progestins that are not androgenic may counteract androgen effects in utero in females as well as in males. While this is only an hypothesis, there is some support from studies on rhesus monkeys. Resko (1975) found that genetic male and female rhesus fetuses did not differ greatly in terms of the concentrations of circulating estradiol and dihydrotestosterone. Male fetuses, however, had higher concentrations of testosterone in plasma, and females had higher concentrations of progesterone. He argued that the higher concentrations of progesterone "protected" the female fetus from possible masculinizing effects of estradiol and dihydrotestosterone.

In conclusion, Ehrhardt offered three generalizations: First, gender role behaviors, which by definition are different, though overlapping, between males and females, are subject to influences of prenatal hormones and are determined independently of genetic sex, sex of rearing, or of gender identity. Second, gender identity is always concordant with sex of rearing and is the variable that is most strongly dependent on social environmental factors. Third, the outcome of prenatal virilization or of other disturbances of sexual development is not homosexuality.

## Notes

1. S. Tobet, J. L. Dunlap, and A. A. Gerall: Influence of fetal position on neonatal androgen-induced sterility and on sexual behavior; MS in preparation.
2. C. M. Bierley and W. W. Beatty, unpublished data.
3. Unpublished observations from Goy's laboratory.

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Stone (1) and Beach (2) reported the execution of elements in the feminine copulatory pattern by male rats which were vigorous copulators in the normal masculine fashion. Various lines of evidence suggest the possibility that male rats possess neural mechanisms capable of mediating the mating behavior of the opposite sex, and that this overt behavior pattern may be manifested when the male, in a high state of sexual excitement, is repeatedly mounted and palpated by a vigorous copulator of his own sex.

Arousal of sexual behavior in prepubertally castrated male rats after administration of testosterone propionate (3), and restoration of mating in postpubertal castrates by the same method (4) indicates that sexual excitability adequate to the occurrence of normal mating is dependent upon androgenic substances. Beach (5) found that sexually inexperienced male rats injected with testosterone propionate prior to opportunity for intercourse with the receptive female displayed a tendency to mate with non-receptive female rats, young male rats and guinea pigs. The failure of most untreated control males to mate with incentive animals other than the estrous female of their own species indicated that testosterone propionate had raised the sexual excitability of the injected males above the normal level.

Utilizing this technic of producing “hyperexcitable” males, and testing the response of such animals to the copulatory attempts of other members of their own sex, an attempt was made in the present study to determine the presence of the female mating pattern in the male rat.

### Experimental Methods

Eight male rats 100 to 125 days of age were given 2 sex tests to determine the presence or absence of feminine copulatory responses prior to the injection of androgenic material. In each test 2 experimental males and one sexually-receptive female were placed in a circular cage 30 in. in diameter. By allowing each male to copulate with the female several times, but removing the female before ejaculation could occur, the experimenter

attempted to create in both males a state of high sexual excitement. Under these conditions the males usually attempted to mount and palpate each other when the female was not available. Judging each male’s excitability by the frequency and vigor of his attempts to copulate with the other male, test conditions were manipulated to increase excitability to a maximum in both individuals.

After 2 tests spaced two days apart each male was given a series of daily injections of 1.0 mg. of testosterone propionate.<sup>1</sup> On the 9th and 10th days of the experiment when each male had received 5 to 6 mg. of the hormone sex tests were repeated. Daily injections continued and tests were conducted on the 14th and 17th days after the administration of 10 to 13 mg. Two males which showed no tendency to mount other males and no indication of excessive sexual excitability were dropped from the experiment after they had received 13 mg. of testosterone propionate. The 6 animals remaining received an injection of 5 mg. on the 18th day, and a second injection of the same amount on the 22nd day. Each of these animals had received a total of 23 mg. at the conclusion of the injection program. On the 24th and 25th days the males were given a number of sex tests. Experimental technic was kept flexible to permit repeated testing and interchange of pairs when such procedure seemed likely to result in increased excitability of the males under observation.

### Results

Experimental results are summarized in table 3.1. Before the injection of testosterone propionate males showed a limited tendency to mount each other. After 5 to 6 mg. had been administered homosexual mounting increased. Seven of the 8 males were mounted 2 to 35 times but no feminine reactions occurred, and 6 of the 7 males fought when mounted.

After receiving 10 to 13 mg. of the hormone 7 of the 8 males were mounted 2 to 30 times. Two males exhibited lordosis and hopping behavior typical of the sexually-receptive female and a third male displayed hopping without lordosis. Only 3 of the 7 males that

**Table 3.1**

Summary of responses to copulatory attempts of a second male before and after injection of testosterone propionate

		Rat							
		1	2	3	4	5	6	7	8
Before injection	No. tests	2	2	2	2	2	2	2	2
	Times mounted	9	0	0	2	2	1	3	2
	Reactions:								
	Fight	+			+	+	0	+	+
	Lordosis	0			0	0	0	0	0
	Hopping	0			0	0	0	0	0
After receiving 5–6 mg.	Ear movement	0			0	0	0	0	0
	No. tests	2	2	2	2	2	2	2	2
	Times mounted	35	0	3	12	16	2	3	4
	Reactions:								
	Fight	+		+	+	+	+	+	0
	Lordosis	0		0	0	0	0	0	0
After receiving 10–13 mg.	Hopping	0		0	0	0	0	0	0
	Ear movement	0		0	0	0	0	0	0
	No. tests	2	3	6	1	3	5	3	2
	Times mounted	30	8	23	2	30	0	29	6
	Reactions:								
	Fight	0	0	0	+	0		+	+
After receiving 23 mg.	Lordosis	0	7	7	0	0		0	0
	Hopping	0	6	1	0	1		0	0
	Ear movement	0	0	0	0	0		0	0
	No. tests		4	6		6	11	8	8
	Times mounted		18	31		70	10	76	72
	Reactions:								
	Fight		+	0		0	+	+	+
	Lordosis		10	9		16	0	0	3
	Hopping		3	0		0	0	8	0
	Ear movement		0	0		11	0	0	1

were mounted showed fighting behavior. Two males were dropped from the experiment at this point.

After being injected with 23 mg. of testosterone propionate the remaining 6 males were mounted 10 to 76 times. One of the 6 cases showed lordosis and hopping behavior. Two displayed lordosis and ear vibrations characteristic of the estrous female. One exhibited lordosis only, one showed hopping, and the final animal displayed none of the elements of the female copulatory pattern.

Although the various reactions typical of the receptive female did not occur every time a male was mounted they were observed frequently enough to assure their presence in the male's behavior repertoire. *Rat 2* was mounted 26 times and exhibited lordosis in 17 instances. During the final series of tests *male 6* displayed lordosis 16 times and ear vibrations 11 times.

The feminine responses shown by injected males were difficult to elicit, sluggishly performed and quickly terminated. The behavior was comparable to that of a female in the early or late stages of estrus.

## Discussion

Present results would be more satisfactory if all males had been mounted as frequently in the preinjection

tests as they were in tests after hormone administration. We might suspect that the feminine mating reactions depended upon the number of times a male was mounted rather than upon the effects of the hormone. In an attempt to check the possibility 4 non-injected males were given 3 sex tests with vigorous copulators and receptive females. The 4 males were mounted 88, 66, 48, and 22 times respectively. In no instance was any feminine behavior displayed by the mounted males.

Table 1 contains additional data indicating that feminine mating reactions are not purely a function of the frequency with which a male is mounted. *Male 1* failed to display any of the elements in the female pattern although he was mounted 67 times in the first 6 tests; whereas *male 2* was mounted only 8 times in the first 6 tests and showed lordosis in 7 of the contacts. *Rat 5* did not show lordosis in the first 7 tests although 13 mg. of hormone had been injected and the male was mounted 48 times. After receiving 22 mg. this animal showed lordosis the 27th time he was mounted.

It is important to note that males exhibiting feminine mating reactions displayed in the same tests vigorous masculine copulatory activity when placed with a receptive female. The behavior of such animals is bisexual rather than homosexual. There is no indication of a sex reversal, but rather an intensification of nor-

mal masculine mating behavior accompanied by occasional feminine responses.

Present results suggest several tentative conclusions. (a) Male rats possess neural mechanisms capable of mediating the mating reactions characteristic of the estrous female. (b) Endocrines do not organize this sexual behavior, but raise the excitability of nervous elements responsible for the organization. (c) Testosterone propionate increases the excitability of both male and female patterns in the male rat. (d) If the stimulus situation permits the occurrence of masculine copulatory behavior the testosterone treated male exhibits only the masculine mating reactions. (e) In the absence of opportunity for masculine copulatory reactions the highly excited male may exhibit the female response when mounted and palpated by a second rat.

### Summary

Eight adult male rats were tested before and after the injection of testosterone propionate for the appearance of feminine mating reactions in response to the copulatory attempts of other males. Prior to hormone administration and after the injection of 6 mg. experimental males usually fought vigorously or tried to escape when other males mounted them in copulatory fashion.

After administration of 13 to 23 mg. of testosterone propionate 5 of the 8 males displayed various elements characteristic of the mating pattern of the estrous female rat. Hopping, lordosis, and vibration of the ears were executed by various injected rats when they were mounted and palpated by other males. The feminine reactions were difficult to elicit and sluggishly performed, but they occurred with sufficient frequency to assure their presence in the behavior repertoire of the experimental animals.

The mating behavior of testosterone treated males is regarded as bisexual rather than homosexual because they displayed very strong masculine copulatory tendencies concomitantly with the appearance of the feminine reactions. It is concluded that the male rat possesses nervous mechanisms capable of mediating the mating pattern of either sex, that testosterone propionate increases the excitability of both patterns, and that within definite limits the stimulus situation is the selective factor determining which type of response will be manifested.

### Notes

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Assistance in making daily injections and in the conduction of sex tests was rendered by Miss Priscilla Rasquin.

1. Crystalline testosterone propionate in sesame oil (5 mg. per cc.), in the form of Perandren, was supplied by Ciba Pharmaceutical Products, Inc., Summit, N.J.

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Certain female animals are known to display mating reactions characteristic of the male of their species. Behavior of this type has been observed in the case of female pigeons (1), night herons (2), rabbits (3, 4), guinea pigs (5), cats (8), pigs (7, 8), cattle (9, 10), monkeys (11, 12), and chimpanzees (13). Despite the wide spread character of such “reversals” of sexual behavior, exhibition of masculine mating behavior by experimentally altered females is frequently offered as evidence to substantiate claims of induced sex reversal (14–19).

In the case of the female rat, masculine copulatory behavior has always been regarded as either nonexistent or quite rare (17, 20, 21–24). Previously the senior author (25) described 7 virgin female rats which mounted sexually-sluggish males. More recently two experienced females have been observed to exhibit the complete masculine copulatory pattern exclusive of the ejaculatory response (26). In all of these instances the females were in estrus and readily accepted the male in typical feminine fashion.

The present study was conducted to determine the proportion of female rats showing masculine copulatory behavior, the frequency of such reactions in each individual, and the relationship of masculine responses to the female’s estrous cycle.

## Methods

Twenty virgin female rats, 3 to 6 months old, raised in segregation were tested for masculine and feminine copulatory behavior. Five animals were discarded before the completion of the experiment because they showed little or no male behavior; and the remaining 15 rats were given daily sex tests throughout 4 estrous cycles. Vaginal smears were analyzed before each test. The experimental female was allowed a short period of adaptation in the testing cage and then a sexually receptive female was introduced. At the end of 5 minutes the receptive female was removed. A vasectomized but sexually vigorous male was placed in the cage and left with the experimental female until he either copulated or attempted to copulate 3 times. The receptive female was re-introduced and the 3 rats remained together in

the cage until 5 minutes from the introduction of the male. The male was then removed and the experimental and receptive females were observed together for another 5 minutes. The daily testing period for each rat thus consumed 15 minutes and there was ample opportunity to observe and record both the masculine and feminine mating behavior shown by the experimental animal.

The masculine copulatory behavior of the experimental females was divided into 3 types of responses: (a) *reaction 1*, sexual clasp in which the mounting rat clasps a second animal with the forepaws about the lateral lumbar region; (b) *reaction 2*, palpation with pelvic thrusts, which consists of mounting, palpating with forepaws and executing pelvic thrusts; and (c) *reaction 3*, complete pattern which includes mounting, palpating and showing pelvic thrusts, giving a final forceful thrust and dismounting with a pronounced backward lunge. The complete pattern is frequently followed by licking of the genitals in a manner typical of the copulating male. In previously published studies of the male rat’s mating behavior *reaction 2* has been called an “attempt” and *reaction 3* has been referred to as a “copulation.” A fourth response shown by males but not dealt with here is the “plug reaction” which accompanies ejaculation.

## Results

### Frequency of Masculine Responses

Certain elements of the masculine copulatory pattern are commonly exhibited by the majority of the female rats in our experimental colony. Of 20 virgin females raised in segregation 18 executed the sexual clasp. Fifteen animals showed palpation and pelvic thrusts; 5 females displayed the complete copulatory response. Five cases which showed only the sexual clasp were not tested extensively. The remaining 15 females received from 17 to 40 tests, the results of which are summarized in table 4.1 and figure 4.1.

The sexual clasp, which was exhibited by each of the 15 animals, appeared in an average of 76 per cent of the tests. During those tests in which this response

**Table 4.1**  
Frequency with which each female exhibited the three types of masculine copulatory responses

Rat	Sexual Clasp					Palpation and Pelvic Thrusts					Complete Pattern				
	Total Tests Given	No. Tests in Which Response Appeared	% Tests in Which Response Appeared	Times Response Was Executed	Average Frequency of	No. Tests in Which Response Appeared	% Tests in Which Response Appeared	Times Response Was Executed	Average Frequency of Re-	No. Tests in Which Response Appeared	% Tests in Which Response Appeared	Times Response Was Executed	Average Frequency of Response Per Positive Test		
					Response Per Positive Test				sponse Per Positive Test						
3	32	17	63	60	3.5	8	34	31	3.9	1	3	2	2.0		
5	28	22	79	102	4.6	6	21	11	1.8	0	0	0	0		
9	19	15	79	67	4.5	10	53	45	4.5	0	0	0	0		
31	19	15	79	83	5.5	7	37	23	3.3	0	0	0	0		
33	33	19	58	50	2.6	16	48	34	2.1	1	3	2	2.0		
34	18	13	72	42	3.1	0	0	0	0	0	0	0	0		
35	40	24	60	123	5.1	15	28	46	3.1	3	8	4	1.3		
36	19	14	74	43	3.1	5	26	16	3.2	0	0	0	0		
37	29	28	97	114	4.1	6	21	19	3.2	0	0	0	0		
38	32	22	72	62	2.8	14	44	35	2.5	0	0	0	0		
39	21	18	86	65	3.6	4	19	4	1.0	0	0	0	0		
42	29	21	72	73	3.5	5	17	6	1.2	0	0	0	0		
43	28	23	82	143	6.2	10	36	45	4.5	2	7	3	1.5		
44	17	17	100	77	4.5	11	65	53	4.8	1	6	1	1.0		
45	26	18	69	104	5.8	8	30	19	2.4	0	0	0	0		
Total	390	286	1142	1208	62.7	125	479	387	41.5	8	27	12	7.8		
Mean	26	19.1	76.1	80.5	4.2	8.9	34.2	27.6	3.0	1.6	5.4	2.4	1.6		

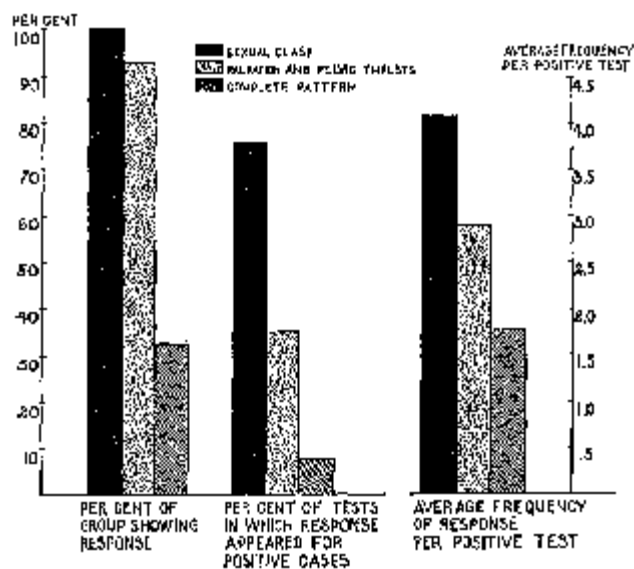


Figure 4.1

Proportion of 15 females exhibiting each type of masculine response and average frequency of each reaction.

occurred it was executed an average of 4.2 times per test. Palpation with pelvic thrusts was shown by 14 females, and these animals exhibited this behavior in an average of 34 per cent of their tests. When the reaction was shown it occurred an average of 3 times per test. The complete copulatory pattern, executed by 5 of the 15 rats, was present in an average of 33 per cent of their tests. During those tests in which it appeared, the complete pattern was displayed on an average of 1.6 times per test.

#### Effects of Repeated Testing

The high percentage of females exhibiting masculine mating reactions, and the frequency with which such responses could be elicited in the animals studied were difficult to reconcile with the general agreement of earlier workers that this form of behavior is rare in the rat. Furthermore, preliminary inspection of the results suggested that masculine reactions increased as testing progressed. It appeared possible that our findings might be strongly affected by the techniques of the present study. These techniques involved repeated presentation of "receptive stimulus" females to the experimental females, the occurrence of occasional pseudopregnancies of experimental animals caused by taking vaginal smears, and by copulation with vasectomized males, and perhaps still other undetected or seemingly extraneous factors. If such influences raised the frequency of masculine responses one might logically expect an increase in male behavior in successive tests.

Comparisons between results of tests conducted during the first and second halves of the experiment

Table 4.2

Frequency of each type of masculine response shown by 15 female rats at various stages in the test series

	Average Number of Responses Per Test		
	Sexual Clasp	Palpation and Pelvic Thrusts	Complete Pattern
First 199 tests	2.3	.7	.05
Last 191 tests	3.4	1.3	.03
First 5 tests	1.9	.4	0
Remaining tests (M = 20.8 per rat)	3.6	1.1	.04

revealed some evidence of increased masculine activity during later tests. In the first 199 tests 15 females displayed masculine reactions a total of 701 times; whereas the same behavior appeared 890 times during the last 191 tests of these animals. Somewhat greater differences appear if we compare the average performance during the first 5 tests with that shown in all subsequent tests. Data presented in table 4.2 illustrate these points.

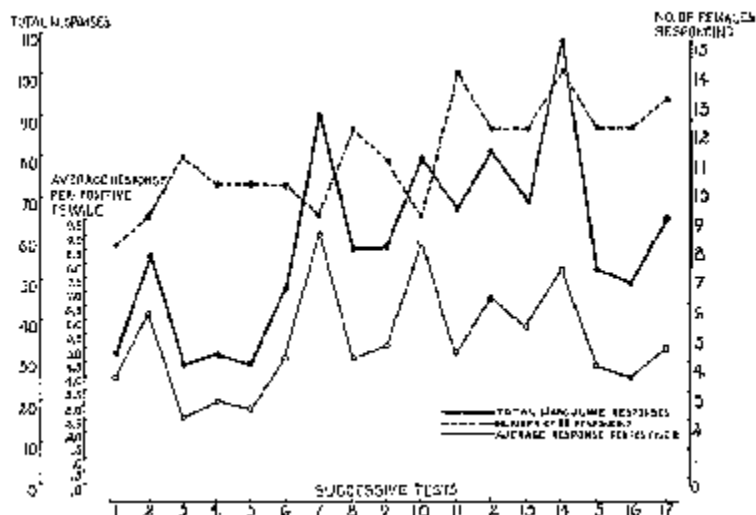
Group records for the first 17 tests which are shown in figure 4.2 reveal that as the experiment progressed there was a tendency for more and more females to show masculine mating responses. The first appearance of male behavior occurred in the first test for 8 females, in the second test for 4 females, in the third test for one female and in the fourth test for one female. In figure 4.2 there is also some evidence of a gradual increase in the number of responses shown by the same female in successive tests (average responses per positive female).

Further indication that continued testing increases the female rat's tendency to show masculine mating reactions is found in data presented in table 4.3. The sexual clasp occurred very early in the testing series for all 15 animals. Palpation with pelvic thrusts appeared somewhat later, after approximately 5 tests. The complete pattern, which was the last response to appear, usually did not occur before the twelfth test.

Repeated testing apparently resulted in some increase in the masculine behavior shown by our females, but the magnitude of the increase is relatively slight, and we feel that the reactions under examination cannot be regarded as artifacts produced by the experimental methods.

#### Feminine Mating Responses in Experimental Females

Although casual observation indicated that the females used in the present work displayed normal feminine receptivity when in heat, this point was checked quantitatively. Data presented in table 4.4 indicate that 11 of the 15 females were most receptive when the vaginal smear contained a preponderance of cornified epithelium. Four of these 11 females were also receptive at



**Figure 4.2**  
Masculine responses shown by 15 females in 17 successive tests.

**Table 4.3**  
Point in the test series at which each type of masculine response first appeared

Response	Number of Females Showing Response	Number of Test in Which Response First Appeared		
		Earliest Instance	Latest Instance	Average
Sexual clasp	15	1st test	4th test	1.7
Palpation and pelvic thrusts	14	1st test	6th test	5.5
Complete pattern	5	6th test	24th test	12.6

other stages in the cycle. One animal was most receptive when the vaginal smear showed chiefly nucleated epithelium; another rat was most receptive in the transitional stage (late nucleated or early cornified epithelial cells). With the exception of *rat 45* these 13 animals exhibited heat behavior as readily and as frequently as the stock breeders from our colony.

It should be emphasized that no attempt was made to determine maximum receptivity, and tests were not spaced so as to occur at any particular stage in the estrous cycle. Accordingly, it is safe to assume that the data presented represent less than the highest degree of heat behavior of which the females were capable. The chances of catching any single animal just at the peak of the receptive period were slight. It is possible therefore that *rats 5* and *44* would have exhibited receptive behavior in a testing series designed to measure responsiveness at every point in the cycle.

The relationship between vaginal smears and receptive behavior in those females which accepted the male can be illustrated roughly by two comparisons. During either the nucleated, transitional or cornified stages of vaginal estrus the 13 animals the receptive behavior of which is shown in table 4.4 received a total of 81

tests (range 2–11). In the course of these tests the females were mounted by males 748 times (range 17–94), and showed lordosis 327 times (range 1–72). Approximately 44 per cent of the instances of mounting by the males elicited lordosis. In the same tests the females exhibited the ear-wiggling reaction 323 times (range 0–104), and the hopping response 233 times (range 0–88). These same females received a total of 155 tests (range 7–24) during periods when the vaginal smears showed over 75 per cent leukocytes. In the course of these tests the females were mounted by vigorous males a total of 1,148 times (range 22–221), but none of the animals displayed lordosis, ear-wiggling, or hopping reactions.

Degrees of receptivity exhibited at different stages in vaginal estrus are further revealed by data presented in table 4.5. The important features of these data are (a) the close parallel between distribution of tests and distribution of the attempts of the male rats to copulate with the experimental female, and (b), the lack of relationship between the foregoing variables and the distribution of the execution by the females of the receptive response (lordosis). For example, 21 per cent of the total number of tests occurred during the cornified stage,

**Table 4.4**

Receptive behavior of 15 females which were also observed for masculine responses

Rat No.	Total Tests During Most Receptive Stage	Times Female Mounted by Male	Times Female Showed Lordosis	% of Mounts Eliciting Lordosis	Total Earwiggle Responses	Total Hopping Responses	Notes
<i>Most receptive during cornified stage of vaginal estrus</i>							
9	6	34	11	32	6	6	Also receptive in nucleated stage
31	6	94	16	17	0	1	
34	5	42	22	52	17	17	
35	7	92	72	78	54	38	
36	2	17	8	47	6	7	Also receptive in nucleated and transitional stage
37	7	69	31	45	49	33	
38	8	36	12	33	7	2	
39	6	55	19	35	6	6	
42	4	35	27	77	19	14	Also receptive in transitional stage
43	8	85	30	35	26	10	Also receptive in nucleated stage
45	6	46	1	2	5	0	Receptivity dubious
<i>Most receptive during nucleated stage of vaginal estrus</i>							
3	11	89	56	63	104	88	
<i>Most receptive during transitional stage of vaginal estrus</i>							
33	5	54	22	40	24	11	Also receptive in nucleated and transitional stages
<i>Never receptive</i>							
5	28	148	0	0	0	0	
44	13	76	0	0	0	0	

**Table 4.5**

Frequency of receptive behavior shown by 13 females at each stage of vaginal estrous cycle

	Type of Cells Predominating in Vaginal Smear			
	Over 75% Nucleated	Over 75% Cornified	Transitional	Over 75% Leukocytes
Percentage of 345 tests occurring at each stage of the cycle	19	21	15	45
Percentage of 2762 mounts by males occurring at each stage of the cycle	19	24	15	42
Percentage of 415 lordosis responses by females occurring at each stage of the cycle	26	63	11	0

and 24 per cent of the males' attempts to copulate took place during this same period; but 63 per cent of the 415 lordosis responses of the females were concentrated in this stage of the cycle. Conversely, 45 per cent of the 345 tests occurred during diestrus (over 75% leukocytes), and 42 per cent of the 2,762 copulatory attempts of the males appeared in the same tests; but not a single lordosis response was elicited from any female during these tests. Data presented in table 4.5 merely repeat what has long been known, that the female rat's display of receptive behavior is closely correlated with vaginal estrus; but they are shown here as a basis for comparisons with the masculine copulatory behavior of the female to be discussed below.

#### Relation of Masculine Behavior to Estrus

In earlier reports (25, 26) the senior author described masculine copulatory reactions shown by both virgin and sexually experienced female rats. In all instances, the females in question were in estrus, and displayed normal feminine receptivity when approached by the male.

Using the results of the present experiment, an attempt was made to compare the frequency of masculine sexual responses, shown during tests in which the experimental females were receptive, with the frequency of the same responses during tests in which there was no indication of feminine receptivity. A female was scored as "receptive" if she showed lordosis in response to at least one third of the male's mounts (i.e., if 33 per cent of the male's mounts elicited lordosis). Nonreceptivity was indicated by refusal to exhibit lordosis. Tests in which receptive behavior occurred once or twice were not counted in either category. The records of 3 females that never showed a high degree of receptivity were excluded from these calculations. Data presented in table 4.6 refer to behavior occurring during the first and final period of the test, at which times the experimental female was in the cage with a "receptive stimulus" female and the male was not present (see description of experimental methods).

Our findings as shown in table 4.6 reveal a tendency for more frequent appearance of masculine reactions during tests in which females were not sexually receptive. The critical ratio  $(m_1 - m_2)/\sqrt{\sigma_1^2 + \sigma_2^2}$  of the difference between the average masculine response per test under the two conditions was 2.96, which suggests the existence of a true difference. Detailed analysis showed that this tendency applied equally to all 3 types of masculine response studied.

In view of the negative relationship between receptivity and frequency of masculine copulatory reactions, it was deemed advisable to attempt to correlate the appearance of masculine behavior with the types of cells present in the vaginal smear. The results of this anal-

**Table 4.6**

Comparison of masculine copulatory responses shown by 12 females during tests in which they were highly receptive and tests in which they were totally non-receptive

Condition of Female	Total Tests in Each Condition	Sum of Masculine Responses	Average Masculine Response Per Test
Very receptive	40	78	1.95
Non-receptive	265	265	3.35

ysis are presented in tables 4.7 and 4.8. Both of these tables show conclusively that the masculine mating reactions of the experimental females were quite evenly distributed throughout all stages of the vaginal estrous cycle.

As may be seen in table 4.7, male behavior appeared in approximately the same proportion of tests (73 to 80 per cent) regardless of the stage of vaginal estrus during which those tests were given. Seventy-four tests occurred when smears contained over 75 per cent of nucleated epithelial cells, and 177 tests were given during periods when smears showed over 75 per cent leukocytes. Masculine copulatory reactions were exhibited in 73 per cent of the first and 72 per cent of the second group of tests. Analyzing the relationships in another way, it is shown in table 4.8 that the distribution of masculine responses among the various stages of vaginal estrus follows almost exactly the distribution of tests given over the same stages.

The difference between male and female copulatory responses shown by the 15 females at each point in the estrous cycle is further illustrated in figure 4.3. The distribution of tests (heavy line) is followed closely by the distribution of the masculine reactions of the females (broken line), and by the distribution of copulatory attempts on the part of the stimulus males (dotted line). This is to say that masculine mating responses by both experimental females and stimulus males vary directly with the frequency of tests, and that this relationship does not fluctuate with changes in the vaginal smear picture.<sup>1</sup> In contrast, the distribution of receptive behavior on the part of the experimental females is closely related to the type of smear, and is relatively independent of either the frequency of the tests or copulatory attempts executed by stimulus males (light line).

#### Behavior of Ovariectomized Adult Females

The apparent lack of relationship between the vaginal estrous cycle and the appearance or frequency of masculine copulatory performance by the experimental females led us to believe that this behavior might be independent of ovarian hormones. As an initial test of this hypothesis 5 of the females were ovariectomized, set aside for from 21 to 43 days, and then re-tested for

**Table 4.7**

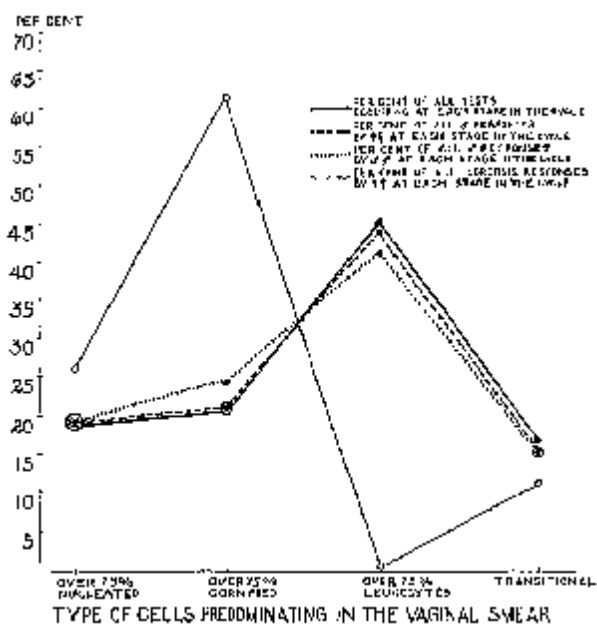
Percentage of tests in which masculine responses occurred at each stage of the vaginal cycle, 15 females

	Type of Cells Predominating in the Vaginal Smear			
	Over 75% Nucleated	Over 75% Cornified	Over 75% Leukocytes	Transitional
Total number of tests occurring in each stage	74	83	177	56
Per cent of tests in which masculine behavior appeared	73	76	72	80

**Table 4.8**

Relative proportions of all tests and of all masculine responses occurring at each stage of the vaginal cycle, 15 females

	Type of Cells Predominating in the Vaginal Smear			
	Over 75% Nucleated	Over 75% Cornified	Over 75% Leukocytes	Transitional
Percentage of the total of 390 tests occurring in each stage of vaginal estrus	19	21	45	15
Percentage of the total of 1,608 masculine responses occurring in each stage of vaginal estrus	19	21	44	16

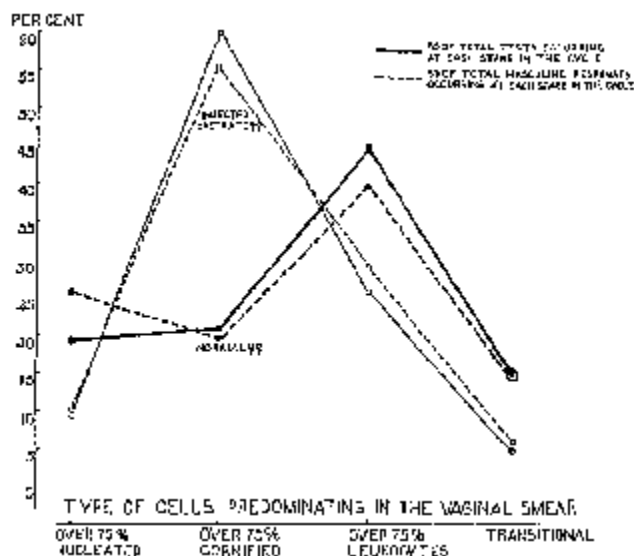
**Figure 4.3**

Distribution of tests, male's copulatory behavior, females' masculine responses and females' receptive behavior over the 4 stages of the vaginal estrous cycle.

male sexual behavior. Prior to castration the 5 animals were given a total of 121 tests, in 84 (69 per cent) of which masculine reactions occurred. After removal of the ovaries the females received 10 tests, and male behavior occurred in all but one test (90%). The average number of masculine responses per test before ovariectomy was 4.6 per test (390 responses in 84 positive tests). After castration there occurred 458 responses in 92 tests, or an average of 5 per positive test.

To re-check the tentative conclusion that there is no relationship between vaginal estrus and the appearance of masculine mating reactions, each of the 5 ovariectomized females was brought into estrus twice. An injection of 500 R.U. of estradiol benzoate was followed 48 hours later by injection of .5 mg. of progesterone.<sup>2</sup> These heavy dosages produced complete cornification for several days and all females became sexually receptive, although not intensely so.

Injection of estrogen and progesterone and consequent induction of estrus had no pronounced effect upon the tendency of the castrated females to show masculine copulatory reactions. The percentage of tests in which male behavior occurred was somewhat higher after castration than before. Prior to ovariectomy masculine responses were shown in 69 per cent of 121 tests. Following operation and the injection of ovarian hormones 81 per cent of 113 tests revealed the execution of such responses. However, this post-castration increase in percentage of positive tests cannot be attributed to the injected hormones for it was apparent in 10 tests given after operation but before hormone treatment (9 of 10 tests positive).



**Figure 4.4**

Distribution of tests and masculine responses over the 4 stages of the vaginal estrous cycle for 5 females tested as normals and then as injected castrates.

The increase in masculine sexual behavior was independent of variations in the vaginal condition. In figure 4.4 the percentage of all tests occurring at each stage in the natural and induced vaginal cycles is represented by solid lines, and the percentage of all masculine responses shown during these same stages is shown by broken lines. In both the natural and artificially created vaginal estrus the distribution of masculine responses closely follows that of tests, indicating an absence of correlation between cycle stages and the tendency to exhibit masculine behavior.<sup>3</sup>

It is known that repeated testing with receptive females presented under constant conditions has a marked effect upon the sexual responsiveness of males of many species (27). A male accustomed to receiving receptive females in a certain cage may attempt to mate with any animal of proper size that is offered in that particular cage. The castrated females described above had received "receptive stimulus" females in the test cage during many preoperative tests, and it appeared possible that the resultant "conditioning" might have contributed to the survival of masculine performance after loss of the ovaries. To test this possibility 5 virgin females from the colony were ovariectomized and examined for masculine copulatory behavior 30 days after operation.

In 10 tests, 4 of the 5 castrates exhibited a total of 134 masculine copulatory reactions (95 clasp, 38 palpate and thrust, 1 complete pattern). The proportion of the small group showing this behavior, and the frequency with which each type of response was executed demonstrated that females castrated in adulthood but

without the experience of repeated sex tests were comparable to experienced castrates in the tendency to exhibit elements of the masculine mating pattern.

In the series of 10 tests, the male behavior of these 4 castrated females showed some tendency to increase with experience. The total number of masculine responses in each succeeding test were as follows: 2, 0, 1, 6, 18, 30, 14, 12, 19, 32. The sexual clasp was shown by the 4 animals after an average of 3.5 tests; palpation with pelvic thrusts first occurred after an average of 5.2 tests; and the complete pattern appeared in the 9th test. Repeated testing seemed to affect the masculine behavior of castrated females in much the same fashion as it did that of intact animals.

#### Behavior of Prepubertally-Ovariectomized Females

Before concluding that the ovaries are not essential to the execution of masculine mating behavior by female rats we considered the possibility that ovarian hormones secreted after puberty but prior to castration might exert some effect permitting the occurrence of male behavior in the adult female after ovariectomy. There was a hypothetical possibility that the nervous system is permanently "sensitized" (in Steinachian terminology) by postpubertal ovarian hormones; and that once such sensitization is achieved the hormones responsible are no longer needed.

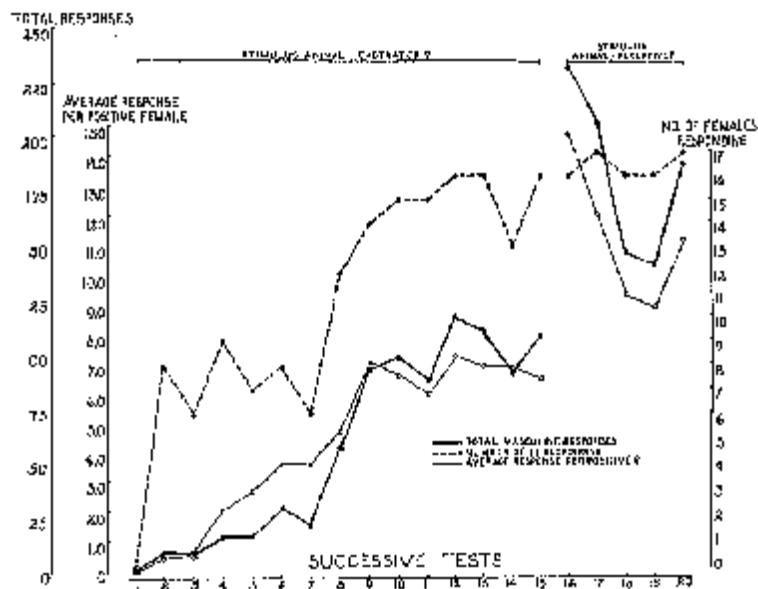
To test this hypothesis, 17 prepubertal female rats were ovariectomized at 21 to 27 days of age and later observed for male behavior. These animals were raised together in one large cage from 21 days of age, and thus from the day of weaning had no contact with males or intact females. Beginning approximately 3 weeks after castration each of the operated females was given a series of fifteen 10-minute sex tests with a cage mate (also prepubertally ovariectomized). When they were 61 to 67 days old the castrates were given five 10-minute tests with a receptive female. Males were never used in this test series.

The results of tests with ovariectomized cage-mates and with receptive females are shown in table 4.9. Masculine copulatory reactions were observed 1,787 times. Of this total, 1,414 responses consisted of the sexual clasp, 361 palpation with pelvic thrusts, and 14 the complete pattern. Test-to-test records for the group as presented in table 4.9 are illustrated graphically in figure 4.5.

Three main trends are observable in the results of the first 15 tests, during which young castrates were tested together in pairs. As the experiment progressed there was an increase in the proportion of the group which showed masculine behavior. The increase in the number of females responding positively resulted in a concomitant rise in the total masculine responses occurring in each test. At the same time the average

**Table 4.9**  
Masculine copulatory responses shown by 17 prepubertally ovariectomized females

Successive Tests	Number of Females Showing Masculine Responses	Total Masculine Responses	Average Number of Masculine Responses Per Positive Female Per Positive Test	Total of Each Type of Masculine Response		
				Sexual Clasp	Palpation and Pelvic Thrusts	Complete Pattern
With castrated female as stimulus animal						
1	0	0	0	0	0	0
2	8	11	1.4	11	0	0
3	6	9	1.5	9	0	0
4	9	18	2.0	17	1	0
5	7	18	2.6	17	1	0
6	8	28	3.5	24	4	0
7	6	21	3.5	21	0	0
8	12	56	4.6	55	1	0
9	14	96	6.9	81	15	0
10	15	99	6.6	87	12	0
11	15	88	5.9	84	4	0
12	16	117	7.3	103	14	0
13	16	110	6.9	97	13	0
14	13	89	6.8	78	11	0
15	16	104	6.5	94	10	0
With receptive female as stimulus animal						
16	16	235	14.7	158	74	3
17	17	206	12.1	145	54	7
18	16	148	9.3	101	46	1
19	16	143	8.9	103	39	1
20	17	191	11.2	129	62	2



**Figure 4.5**  
Masculine responses shown by 17 prepubertally castrated females in 20 successive tests.

**Table 4.10**

Age of castrated females at test in which masculine responses first appeared, 17 females

Number of Cases	Days of Age at First Test	Average Number of the Test in Which First Masculine Response Appeared	Average Days of Age at Test in Which First Masculine Response Appeared
2	43	3.0	45.5
8	40	4.4	44.1
4	39	4.3	43.0
3	37	6.7	44.0

number of masculine responses per positive female showed a fairly steady increase in successive tests.

These various trends are susceptible to several explanations. (a). Results obtained in successive tests with intact or castrated adult females revealed a tendency toward an increase in the number of females responding and in the average number of responses per positive female per test. It is permissible therefore to regard experience in the testing situation as one factor contributing to the progressive increase in responsiveness shown by prepubertal castrates.

(b). All 17 of the young animals were tested on the same days, and since there was a 6-day variation in date of birth, the females were different ages at the time of the first test. That these differences in age may have influenced the results is revealed by data presented in table 4.10. The oldest females tended to show masculine responses earlier in the test series than did younger rats. The second and third columns in table 4.10 suggest an inverse relationship between age at the time of the first test and the number of the test in which masculine behavior first appeared. The fourth column indicates that such behavior usually first occurred at 43 to 45 days of age. Therefore part of the increase in masculine responses shown in table 4.9 and figure 4.4 may be interpreted as reflecting this function of the increasing age of the test animals.<sup>4</sup>

In tests 16 to 20 the prepubertal castrates were given receptive females as stimulus animals. Table 4.9 and figure 4.5 show that this change in experimental conditions resulted in an abrupt and marked increase in masculine mating responses shown by the experimental females. In tests with receptive females the prepubertal castrates showed an increase in the number of sexual clasps, and in palpation with pelvic thrusts; and the complete pattern, which had not been elicited in any of the preceding tests, appeared during this period.

Table 4.11 presents comparisons between the masculine copulatory reactions shown in response to a receptive female by intact adult females, adults castrated before experience in sex tests, and prepubertal cas-

**Table 4.11**

Comparisons of masculine copulatory behavior shown by intact adult females, castrated adult females and prepubertally castrated female rats

Group	Stimulus Animal	Number of Tests Per Female	Sexual Clasp			Palpation and Pelvic Thrusts			Complete Pattern		
			% of Group Responding	% of Tests in Which Positive Cases Showed the Response	Average Frequency of Response Per Positive Test	% of Group Responding	% of Tests in Which Positive Cases Showed the Response	Average Frequency of Response Per Positive Test	% of Group Responding	% of Tests in Which Positive Cases Showed the Response	Average Frequency of Response Per Positive Test
15 intact adults	receptive female	20	100	76	4.18	93	34	2.96	27 <sup>1</sup>	5	1.57
5 adult castrates	receptive female	10	80	55	4.32	80	28	3.45	20	10	1.00
17 prepubertal	receptive female	5	100	95	7.63	77	79	4.83	24	40	1.30
17 prepubertal castrates	prepubertally castrated female	15	100	92	4.51	53	32	2.14	0	0	0

<sup>1</sup> Subsequent tests raised this figure to 33.

trates. Also included in this table are the records of the prepubertal castrates in tests when the stimulus animal was a second prepubertally castrated female. Variability in the size of the groups, and in the number of tests given undoubtedly contributes to some of the intra-group differences in responsiveness to the receptive female. Differences which seem large enough to be significant can be traced to variation in the ages of the experimental groups.

All females with the exception of one adult castrate showed the sexual clasp; but juvenile castrates showed this response in a much higher proportion of the tests than did adult females. This difference cannot be due to ovariectomy for the adult castrates were even less responsive than intact rats of comparable age. During those tests in which the sexual clasp occurred it was executed nearly twice as frequently by the prepubertal castrates as by females in the other two groups. Again, the variation seems traceable to an age factor rather than absence of ovaries since the record of adult castrates approximates that of intact adults. In comparison with normal adults, a somewhat lower percentage of the young castrates showed the palpate and thrust response, but those juvenile females which did exhibit this reaction executed it in a much higher proportion of the tests and a greater number of times per positive test than did the adult females.

Approximately the same proportions of the 3 groups showed the complete pattern, and the frequency of its execution during tests in which it did appear was about the same for all groups. However those prepubertal castrates which displayed the complete pattern tended to do so in 40 per cent of the tests, whereas adult animals responded with this reaction in only 5 to 10 per cent of all tests.

Comparison of the reactions of prepubertal castrates to another castrate and to a receptive female reveals that the latter was uniformly a more effective stimulus animal. When a receptive female was presented, each type of masculine copulatory response occurred in a higher percentage of tests and was exhibited more frequently during the positive tests.

## Discussion

The findings presented above are at variance with earlier reports in several respects. Other experimenters have regarded the execution of masculine copulatory reactions by female rats as very rare, whereas 18 of the 20 intact adult females which we have described showed such behavior. This apparent contradiction is undoubtedly due in part to the fact that nearly all of the previous reports have been based upon casual observation under uncontrolled conditions. The behavior in question probably appears much more frequently

when 2 females are repeatedly tested together than when a number of females, or males and females are kept together in a large cage and observed incidentally.

We have interpreted as masculine behavior the execution of single elements which make up the copulating male's total response. Other investigators may have observed female rats exhibiting the sexual clasp, or palpation with pelvic thrusts, but preferred to restrict the term "male sex behavior" to the appearance of the reaction which we have designated as the "complete pattern."

A part of the frequency with which females herein reported exhibited masculine responses has been shown to be the result of repeated testing.

There is a possibility that females from our particular colony are more active in a masculine direction than are females from other stock. This aspect of the problem has not yet been checked.

The apparent lack of correlation between ovarian cycles and the appearance of masculine sexual behavior presents several problems. Previously one of us (25, 26), in describing this behavior tended to regard it as "abnormal behavior" associated with estrus. This misconception arose because we observed masculine behavior only in receptive females. However, nonreceptive females were rarely given opportunity to react at all. The masculine behavior was seen in females used as stimulus objects in an experiment upon copulatory behavior of the male rat. Females were put with males at the start of a day's testing. Nonreceptive females were returned to the colony, while receptive animals were placed together in one cage where they were kept when not in use with males. Under such conditions receptive females in the resting cage were seen to mount each other. From such observation it was incorrectly inferred that this behavior was associated with receptivity and therefore with estrus. We had no proof that nonreceptive females would fail to respond similarly, and present results show that they do.

It seems not unlikely that reports of a close relationship between heat and male-like mounting behavior in the female of various mammalian species may reflect similar absence of systematic observation of diestrous females. In fact, there is little indication that any investigator has carefully and repeatedly tested for the appearance of this behavior in diestrous females confronted with a receptive female as the stimulus animal. In general, emphasis has been placed upon masculine responses seen at the time of heat, and evidence for the absence of such responses at other periods is either scanty or totally lacking. It follows, therefore, that the generally accepted belief that masculine mounting behavior is associated with heat, and thus, by inference, dependent upon ovarian hormones, may be the result of purely fortuitous factors.

One must except from the foregoing generalization the studies of Ball (23) and those of Young and his co-workers (5, 28, 29, 30). Ball investigated the effects of testosterone on sexual behavior in the female rat and concluded that untreated females rarely exhibit the male pattern. Ball did note that the normal receptive females (used as stimulus animals) mounted the testosterone-treated females, but interpreted this behavior as due to some particularly stimulating aspect of the behavior of the testosterone-treated animals. Furthermore, Ball reports one instance in which a normal female in diestrus mounted a receptive rat. Thus differences between present findings with intact adult females and those reported by Ball consist merely of lack of agreement as to the frequency of masculine mating reactions in the female. The two studies agree in finding that the responses are present, and that they may be exhibited both during estrus and diestrus.

Observations on female guinea pigs are more difficult to reconcile with present results unless one dismisses the problem by categorizing it as species difference. The female guinea pig's display of mounting behavior is temporally related to receptivity, and Young and Rundlett (28) have suggested the possibility that mounting, like the heat response, is stimulated by the synergistic action of estrogen and progesterone. This hypothesis cannot be applied to our findings with the rat in view of the appearance of masculine responses in adult and prepubertal castrates. It should be noted, however, that, in the guinea pig, heat and mounting activity occasionally occur independently, and when they do appear simultaneously there is no relation between frequency of mounting and length of the heat period (5).

We have no positive evidence to indicate the hormonal basis of masculine mating reactions in the female rat. Obviously ovarian secretions are not essential. If it is assumed that the appearance of male behavior is always dependent upon androgens (a wholly gratuitous assumption) it is possible to speculate on the source of the androgen involved in this instance. The adrenal cortex might be regarded as a possible source of androgens, but speculation on this point seems fruitless at the present stage of knowledge. Certainly there is no reason to associate the behavior under consideration with any organic dysfunction (e.g. cortical adrenal tumor). More probably the display of masculine copulatory responses is to be regarded as typical of female rats from our colony when confronted with a particular stimulus situation.

The negative relationship between receptivity and masculine activity cannot be explained yet to our satisfaction. At first glance there seems to be direct contradiction involved in the finding that receptivity is positively related to vaginal estrus and negatively re-

lated to the appearance of male behavior, while at the same time there is no relation between masculine behavior and vaginal estrus. In an attempt to resolve this apparent conflict we suggest that the positive relationship between vaginal estrus and receptivity is far from a perfect correlation; while the negative relationship between receptivity and male behavior, although showing a statistically significant difference, is not extreme.

From the point of view of psychobiology, it is interesting to note the similarities between the masculine sexual behavior of male and female rats. There are at least two points for comparison. If a group of either males or females is repeatedly tested with receptive females, the proportion of the group exhibiting masculine behavior increases during the first few tests and then remains fairly constant. In groups of either sex there are some animals which are either more easily aroused sexually, or less easily inhibited by the testing situation, and therefore copulate or attempt to copulate with the receptive female in the first tests. A second class of rats fails to show masculine behavior in initial tests but does so later in the test series. A final group, both among males and females, never attempts to mount the receptive female.

A second comparison of the masculine sexual reactions shown by male and female rats lies in the definition of the adequate stimulus pattern. It has been shown that male rats exhibit copulatory behavior most readily and frequently in response to a receptive female. The phenomena of sexual excitability intimately involve sensitivity to a pattern of multisensory stimulation; and in the rat this responsiveness to a particular stimulus pattern appears to be mediated by an inherited nervous organization (27, 31, 32). We have described above an immediate increase in masculine behavior shown by prepubertally castrated females when receptive females were substituted for castrates as the stimulus animal. We interpret this increase as a reflection of heightened sexual excitement on the part of the experimental females. In earlier papers (23, 25, 26) it has been suggested that female rats inherit a neuromuscular organization capable of mediating the copulatory pattern characteristic of the male of their species. Present findings indicate that in the female as in the male this motor pattern is elicited most readily by that stimulus pattern presented by the receptive female.

It is always taken for granted that in many types of instinctive behavior the same stimulus → response relationships exist for both sexes (e.g., hunger → food → eating, or, pain → avoidance reactions). Usually, however, the mating patterns of the sexes have been regarded as sex-specific. It is therefore of definite theoretical interest to observe that when the masculine copulatory response appears, it is linked with

a particular stimulus pattern; and that this relationship of stimulus to response is constant regardless of the sex of the reacting animal. For some purposes the reactions usually regarded as masculine copulatory behavior might better be defined in terms of a stimulus → response relationship with no reference to the sex of the animal showing the behavior.

### Summary

Twenty adult female rats were tested for the appearance of both feminine and masculine copulatory reactions. Eighteen animals showed sexual receptivity when mounted by males during estrus. Eighteen rats responded to receptive females with the execution of certain elements of the masculine copulatory pattern. Three types or grades of mating behavior characteristic of the copulating male and shown by the females herein described are the sexual clasp, clasp with palpation and pelvic thrusts, and the "complete pattern" which includes all of the foregoing plus dismounting after a final, vigorous pelvic thrust and frequently involves licking of the genitals.

The feminine sexual pattern (receptivity) occurred during the period of vaginal estrus and could never be elicited when the smear consisted chiefly of leukocytes. Masculine copulatory reactions were performed with equal frequency at all stages of the estrous cycle. Castration of 5 females eliminated receptive behavior but had no obvious effect upon the execution of the male pattern. Injection of estrogen and progesterone revived receptivity and did not alter masculine behavior.

Seventeen female rats were ovariectomized at 21 to 27 days of age and tested between the ages of 39 and 81 days. All of these juvenile castrates showed the sexual clasp. Thirteen animals clasped with palpation and pelvic thrusts, and 4 females exhibited the complete pattern. When tested with other female castrates and then with a receptive female the prepubertal castrates showed a greater amount of masculine behavior in response to the latter stimulus animal.

It is concluded that the majority of female rats from the colony studied possess a neuromotor organization capable of mediating the masculine copulatory pattern. It is further concluded that in the female this motor pattern appears most readily in response to the same stimulus pattern by which it is evoked in the male, namely a multi-sensory pattern of stimulation presented by the receptive female. Finally, it is concluded that the masculine sexual reactions of the female rat are not dependent upon ovarian hormones.

### Notes

This study was supported by a grant from the Committee for Research on Problems of Sex, National Research Council.

1. The rise in the curve of test frequency at the point of diestrus is occasioned by the temporal span of the various stages of the complete cycle. Since leukocytes predominate for about half of the 4-day cycle the fact that 45 per cent of all tests occurred during this stage is easy to understand.

2. We are indebted to the Schering Corp., Bloomfield, N. J. for the estradiol benzoate (Progynon-B) employed, and to Ciba Pharmaceutical Products, Inc., Summit, N. J. for the progesterone (Lutocylin) used in the present work.

3. The shift toward a majority of tests during cornified stage of induced estrus simply reflects the prolonged cornification resulting from very large doses of estrogen.

4. This evidence should not be taken to mean that masculine copulatory behavior cannot be elicited in female rats prior to 43 days of age. On the contrary, experiments now in progress reveal the appearance of such reactions at a much earlier age. Present results merely suggest that for the particular females investigated, and under the conditions imposed, the responses involved tended to appear at approximately 44 days of age.

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## Introduction

It is 25 years since the death of Geoffrey Harris, and the existence of hypothalamic peptides controlling anterior pituitary secretion has become just another section in an undergraduate textbook. In a reductionist world, where molecular biological techniques are revealing an ever-accelerating number of new genes, the broader picture of the whole animal and its integration with its environment can be something we take perilously for granted. This review attempts to reconstruct some of the mystery and drama of those earlier times, and to show the combination of tenacity and insight that went into the hard-won discovery of what now seems commonplace.

## Seasonal Influences on Breeding

Introducing his Croonian Lecture on “Sexual periodicity and the causes which determine it” to the Royal Society in 1936, Francis Marshall, one of the great pioneers of reproductive physiology, observed:

The great majority of animals, both vertebrate and invertebrate, not to mention plants, have a more or less definite season of the year at which they breed. There is no month of the year at which some species does not have its breeding season, and yet for the particular species in question the season is most regular. In view of the general correlation between the seasonal and the sexual cycles it must be assumed that these stand in the relation of cause to effect (Marshall 1936).

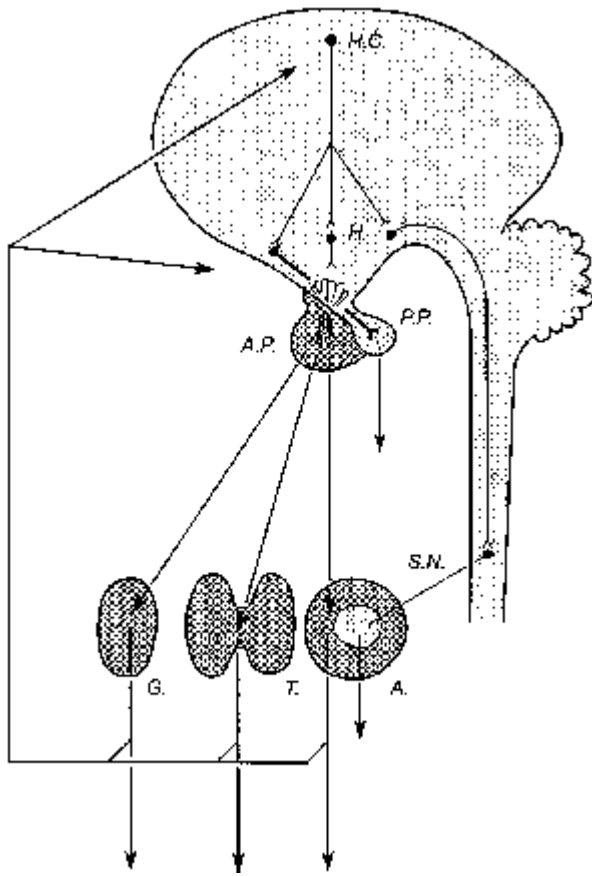
The persuasive simplicity and the breadth of Marshall’s postulate lead at once to the question of mechanism: How do the changing seasons determine the time when an animal breeds? What parts of the body are involved in the recognition of these environmental signals? What are the anatomical and physiological channels for their transmission? And which molecules carry these messages? These questions occupied the life’s work of Geoffrey Harris, from his first contact with Marshall in Cambridge in 1936, to his death in 1971.

## Electrical Stimulation: A Laboratory Model of Environmental Events

To examine the complex natural control mechanisms postulated by Marshall, the first step was to devise a laboratory situation that could mimic the effects of environmental events on reproduction. “Marshall had already formed the opinion,” Harris wrote in his classical 1955 monograph, *Neural Control of the Pituitary Gland*, “that sensory stimuli act through reflex pathways in the central nervous system to affect the secretion of the anterior pituitary gland, which acts as a liaison organ between the nervous and reproductive systems” (figure 5.1) (Harris 1955). Experimental investigations of this liaison began with attempts to simulate environmentally induced events by electrical stimulation.

In rabbits the stimulus of mating induces ovulation. Both ovulation and the maintenance of corpora lutea were known to depend on the secretory function of the anterior pituitary gland (e.g. Smith 1927). In 1936, Marshall and Verney had made the seminal observation that in the absence of mating, diffuse electrical stimulation to the head of female rabbits was sufficient to trigger the mechanism for ovulation and that the ruptured ovarian follicles developed into functioning corpora lutea able to maintain a pseudopregnancy (Marshall & Verney, 1936).

Harris’s first work was carried out in the Department of Anatomy at Cambridge when he was a medical student. Following Marshall and Verney, he applied similar diffuse stimulation to the head of rats (Harris 1936). This species, unlike the rabbit, normally ovulates spontaneously, but unless mating occurs, the ruptured ovarian follicles do not develop into functioning corpora lutea. The effect of the electrical stimulation was that the next ovulation was delayed, but then was followed by a pseudopregnancy. Harris concluded that “excitation of some neural structure had occurred, and this in turn had stimulated the anterior pituitary.” But which structure had been excited?



**Figure 5.1**

Neuro-endocrine integration. This figure, adapted from Harris's monograph of 1955, already shows the global theory of the reciprocal relationship between the CNS and the endocrine system in terms not different from our present concepts. The "higher centres" (H.C., a term that Harris also puts in quotation marks) influence the hypothalamus (H.), from which the portal vessels carry controlling factors to the anterior pituitary (A.P.), whose hormones in turn separately regulate the secretions of the gonad (G.), the thyroid (T.), and the cortex of the adrenal (A.). The hormones of these target glands are shown feeding back at hypothalamic and higher CNS levels. In addition, two nervous control pathways are shown: (a) the supraoptic and paraventricular projections through the pituitary stalk to the posterior pituitary (P.P.) and (b) the projection from the spinal preganglionic neurons through the splanchnic nerves (S.N.) to the adrenal medulla.

"Marshall suggested to me," Harris wrote in his Dale Lecture, published posthumously in 1972, "that it would be interesting to apply precisely localized electrical stimuli to different regions of the hypothalamus of estrous female rabbits to see if it were possible to obtain evidence concerning the reflex pathway normally involved in post-coital ovulation" (Harris 1972).

During the next year, Harris reported that stimulation in the hypothalamus evoked ovulation in the rabbit, and noted (with what he later called "the brashness of the young"): "There is no reason to believe that the thyrotropic, adrenotropic, lactogenic, parathyrotropic and growth hormone are not similarly controlled"

(Harris 1937). Like Luther, he had nailed his articles to the church door, and by the end of his life, he would have demonstrated the truth of some, but by no means all of his postulates.

Over the next 10 years Harris set about devising a technique to apply electrical stimulation to precisely localized areas, but under conditions sufficiently non-invasive that the endocrine system would continue working normally. Taking advantage of the wartime advances in electronics that had been needed for the development of radar, Harris designed a stereotactically placed, chronic indwelling electrode attached to a small secondary induction coil that was inserted under the scalp and activated by a large primary induction coil surrounding the cage (Harris 1947, 1948a,b). These electrodes were able to deliver highly localized stimuli. The electrodes could be used for chronic stimulation in freely moving, unanesthetized rabbits. The method of remote stimulation avoided the stress caused by ether or other anesthetics, which themselves can interfere with ovulation or induce pseudopregnancy and which affect the secretion of adrenocorticotrophic hormone (ACTH) and several other anterior and posterior pituitary hormones.

Over the years, this meticulously developed and surgically demanding technique would serve Harris well. He was to use it to study the control of the release of the anterior pituitary hormones—luteinizing hormone (LH, involved in ovulation), prolactin (involved in pseudopregnancy, pregnancy, and lactation), ACTH, and thyrotrophic hormone (TSH)—and the posterior pituitary hormones—oxytocin and vasopressin.

Because of the precision of localization, Harris was able to show that the crucial position for inducing an ovulatory response was when the electrode tip lay in the hypothalamus, and he could now confirm the observations of Markee et al (1946) that the pituitary gland itself was nonresponsive (Harris 1948a). This led to the important conclusion that the ovulatory signal generated by stimulating the CNS must have passed from the hypothalamus to the pituitary. But the nature of the pathway from the hypothalamus to the anterior pituitary was unknown: The search was now on for the link between the hypothalamus and the pituitary gland. Harris was on the threshold of his first major breakthrough.

### The Neurovascular Link

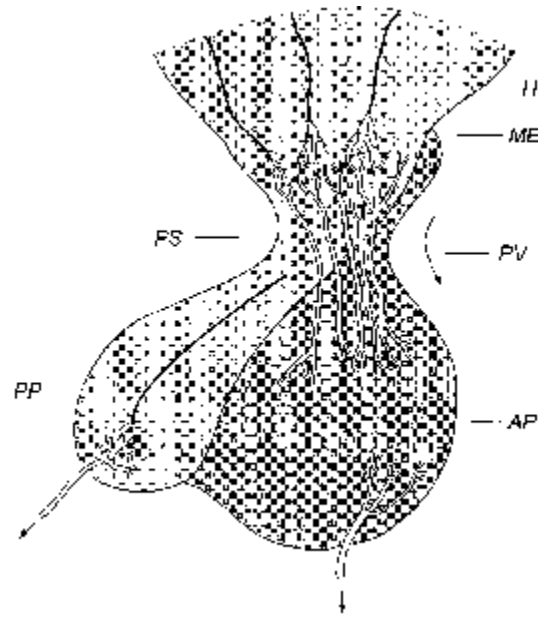
The most obvious link from the nervous system would have been by means of nerve fibers, and for several other endocrine organs a nervous link was already known. Thus, as Harris noted, "the adrenal medulla has a direct nerve supply, which controls its secretion; the posterior pituitary also has a direct nerve supply"

(Harris 1955). Harris used his method of localized electrical excitation to show that unlike the anterior pituitary, the posterior pituitary does respond to localized electrical stimulation by release of vasopressin (causing antidiuresis and an increase in urinary chloride concentration), and oxytocin (causing increased uterine motility, as measured by a balloon) (Harris 1947). Thus the presence of a nerve supply passing down the pituitary stalk from the brain to the posterior pituitary is correlated with the ability to induce secretion by electrical stimulation.

The paradox is that the anterior pituitary, which is crucially involved in the response to environmental, seasonal, and mating stimuli, is clearly under the control of the CNS, but despite considerable efforts to find one, it has scant or no innervation. "The pars distalis of the pituitary," Harris wrote, "may be described as a gland under nervous control but lacking a nerve supply" (Harris 1948b). How then is the nervous system able to control the release of the anterior pituitary hormones?

Not for the last time, chance was to play an important part in Harris's life. Harris's work in the Department of Anatomy at Cambridge coincided with the visit of Professor Gregory T Popa, of the Department of Anatomy at Jassy in Romania. Popa & Fielding (1930, 1933) had shown that the human pituitary stalk is clothed by a system of hypothalamo-hypophysial vessels, which were named portal (by analogy to the hepatic portal circulation) because they were connected at both ends to a capillary plexus—above to the capillary plexus of the median eminence on the under surface of the midline hypothalamus, and below to the capillary plexus of the anterior pituitary gland (figure 5.2). In a striking series of comparative anatomical studies of a large range of different vertebrate species, John D Green, Harris's close friend and fellow demonstrator in anatomy, demonstrated the phylogenetic constancy of the hypophysial portal circulation and the capillary loops in the median eminence (Green 1951). This degree of evolutionary conservation suggested that such an anatomical arrangement must have a very important functional role. In fact, as Harris was to show, the messages carried through this unique area of specialized vasculature were vital for all aspects of reproduction, which from an evolutionary point of view, is the most basic function needed for survival of the species. In 1947, Green & Harris suggested "that the central nervous system regulates the activity of the adenohypophysis by means of a humoral relay through the hypophysial portal vessels" (Green & Harris 1947).

However, for some time after their original discovery, the functional importance of the portal vessels remained obscured by uncertainty about the direction



**Figure 5.2**

The portal vascular link. Hypothalamic (H) nerve endings are shown arborizing in the median eminence (ME), where they release their secreted factors into the primary plexus of the portal vessels (PV), which carry them down the pituitary stalk to be released at a secondary plexus in the anterior pituitary (AP), where they regulate the secretion of the anterior pituitary hormones into the general circulation. In contrast, the posterior pituitary (PP) is directly innervated by hypothalamic fibers passing down the pituitary stalk (PS) and releasing their secretions directly into the general circulation. (Adapted from Fawcett et al 1968.)

of blood flow within them. Popa & Fielding (1930, 1933) believed that the blood flowed from pituitary to brain, whereas Wislocki (1937, 1938) believed that it flowed from brain to pituitary. In amphibia, several investigators had directly observed in living animals that the portal blood flow was from the hypothalamus to the pituitary (e.g. Houssay et al 1935, Green 1947).

Harris initially accepted the view of his visiting senior colleague, Popa, that the blood flow was from the pituitary to the brain (Harris & Popa 1938). But finally, by a combination of india ink perfusion techniques and direct microscopic observation in the living rat, Green & Harris (1949) confirmed Wislocki's finding (1937, 1938) that the portal blood flows from the hypothalamus to the pituitary. This was the first demonstration of portal blood flow in a living mammal. [Later work has shown that in fact there is also a system of vessels with blood flowing in the opposite direction as well (Bergland & Page 1978).]

The portal vessels were, therefore, in a position to provide an anatomical pathway to convey the messages by which electrical stimulation of the hypothalamus could induce the secretion of hormones by the anterior pituitary, and it was therefore possible (as others had speculated, e.g. Hinsey & Markee 1933, Friedgood

1950) that the neural control of pituitary secretion could involve a humoral agent carried through this vascular link. This would explain why electrical stimulation could elicit an ovulatory response from the hypothalamus but not from the anterior pituitary.

Cajal (1911) had described hypothalamic nerve fibers that terminated on the capillaries of the median eminence. In his 1955 monograph, Harris suggested that these nerve endings might contain "some humoral substances which are liberated into the capillaries of the primary plexus of the median eminence, and carried by the portal vessels to excite or inhibit the secretion of the gland cells in the anterior pituitary" (Harris 1955). Later electron microscopy (reviewed in Harris & Campbell 1966) showed that these capillaries are of the specialized fenestrated type found in other secretory and absorptive organs, with a double basal lamina separated by a narrow collagen-containing space. The blood-brain barrier is here permeable to the transmission of larger molecules. The nerve fiber terminals on the outer layer of the basal lamina contain synaptic vesicles and a variety of larger dense-cored vesicles.

The problem for Harris was how to prove that this anatomical arrangement of the hypothalamo-hypophysial portal vascular system did, in fact, constitute the functional link in the hypothalamic control of anterior pituitary secretions. "Sufficient evidence is not available," Harris wrote, "to prove the neurohumoral control of the adenohypophysis, but we feel this theory has much to support it" (Green & Harris 1947).

In Harris's opinion (1955), the definitive proof of a functional link would require observations that were, at that time, technically impossible, viz collection and analysis of the portal capillary blood, identification of the secreted hypothalamic substances (named releasing factors, according to the proposal of Saffran et al 1955) with the required functional specificity, and demonstration that appropriate changes in these factors actually occurred in vivo under the relevant physiological situations (such as ovulation) and that the changes in the releasing factors were both necessary and sufficient to cause functionally effective changes in the rate at which anterior pituitary hormones were secreted into the general circulation. It was only toward the end of Harris's life (see below) that George Fink, working in Harris's laboratory, was able to confirm that these criteria were valid for the hypothalamic factor needed to trigger the release of an ovulatory quota of LH from the anterior pituitary.

#### **The Role of the Hypothalamo-Hypophysial Portal Vessels in Reproduction**

As a first step to validating his proposal that the hypothalamo-hypophysial portal vascular link was es-

sential for the control of anterior pituitary functions, Harris carried out a series of experiments with pituitary stalk section and pituitary transplantation that were designed to show that the normal control of the secretions of the anterior pituitary could occur only if the gland was vascularized from the capillaries of the median eminence.

It had long been known that the pituitary gland was essential for gonadal function. But up to that time, the extensive literature on the effects of pituitary stalk section was conflicting and did not exclude the possibility that the pituitary might function when no longer in contact with the brain. As Harris now showed, this confusion had arisen because of the failure to recognize the regenerative capacity of the portal vessels. In his initial work with Gregory Popa in the rabbit (Harris & Popa 1938) and later in the rat (Harris 1950) and the monkey (Harris & Johnson 1950), Harris developed surgical techniques for cutting the pituitary stalk and demonstrated that cut portal vessels rapidly regenerate, revascularize the pituitary, and restore gonadal function. But if regeneration is prevented by insertion of a waxed paper plate, the gonads remain atrophic (Harris 1950, Fortier et al 1957). This, then, provided a clear indication that the portal vasculature was essential for the gonadotrophic function of the anterior pituitary, but it still remained to be shown that the failure of pituitary secretion after stalk section with prevention of portal vessel regeneration was not due to a general ischemic compromise, but was caused by deprivation of specific secretion-regulating hypothalamic factors.

This task was accomplished by a series of transplantation experiments, carried out after Harris's move to the Department of Physiology at Cambridge. In collaboration with Dora Jacobsohn of the University of Lund in Sweden (Harris & Jacobson 1950, 1952), Harris provided the definitive proof of the functional importance of the hypothalamo-hypophysial portal vascular system in regulating anterior pituitary secretion. It had already been shown (see Harris 1955) that while endocrine glands (such as the testis, adrenal cortex, ovary, and thyroid) can function in a regulated fashion when transplanted into a part of the body remote from their original site (e.g. under the kidney capsule), the anterior pituitary gland does not. Using the surgical parapharyngeal hypophysectomy approach [devised by Philip Smith (1927) to avoid damage to the hypothalamus or any part of the brain], Harris & Jacobsohn (1950, 1952) showed that although anterior pituitary tissue does not function normally if transplanted away from its original site, it will function if transplanted back under the midline hypothalamus within reach of the host portal vessels.

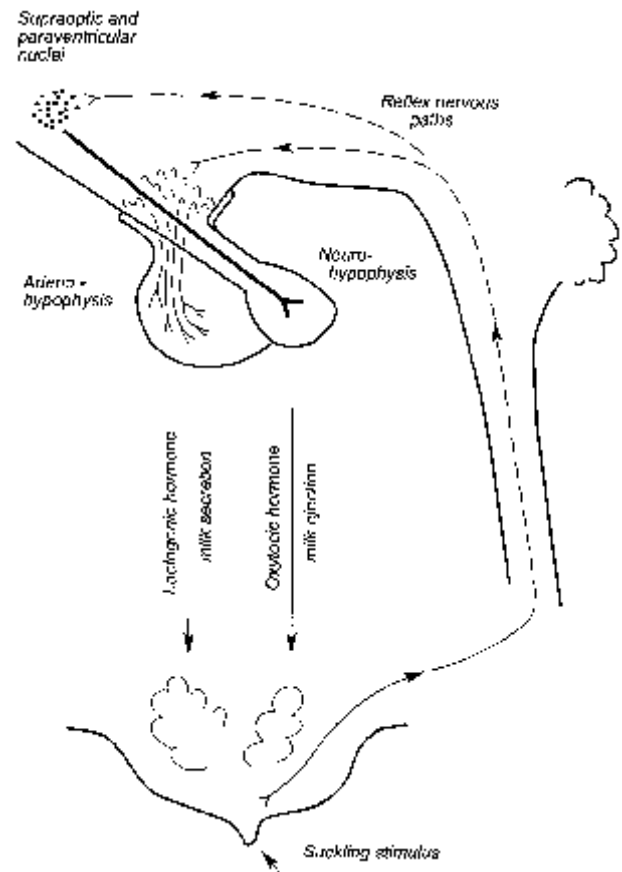
In an extensive physiological analysis of the criteria for successful return of multiple anterior pituitary func-

tions in the rat, Harris & Jacobsohn (1950, 1952) demonstrated that neonatal or adult pituitary donor tissue of either sex could restore cyclic ovulation, mating, pregnancy, and parturition, as well as adrenal weight and functional thyroid histology. Subsequent histology showed that these functions were only resumed in cases where the transplanted pituitary had been revascularized from the primary capillary plexus of portal vessels in the median eminence. Transplants located on the adjacent ventral surface of the brain outside the portal vascular field survived, and even though they became equally well revascularized by nonportal vessels from the temporal lobe, they still did not function. However, pituitary transplants that had been grafted away from their normal site and allowed to establish there in a nonfunctioning state would resume function if retransplanted back into their normal site under the median eminence (Nikitovich-Winer & Everett 1958).

From such observations, Harris proposed that the median eminence is the final common pathway for transducing neural signals into releasing factors, which are carried in the portal vessels to the anterior pituitary, which in turn controls the separate functions of the distant target organs through secretion into the general circulation, thus bringing a wide range of endocrine glands under control of the CNS (figure 5.1).

Posterior pituitary functions, however, were not fully restored in these transplants. After delivery of the young, the mothers produced milk as normal, and the mammary glands became engorged, but there was no let-down of milk, and therefore the litters did not survive. This defect could be remedied by administration of oxytocin, suggesting that the let-down of milk may require a reflex release of oxytocin from the posterior pituitary gland that is induced by the stimulus of suckling (figure 5.3). With his graduate student Barry Cross, Harris confirmed this by showing that electrical stimulation of the supraoptico-hypophysial tract (i.e. the pituitary stalk) induces milk ejection (measured by cannulating a mammary duct) (Cross & Harris 1950) and that this effect is prevented by lesions of the tract (Cross & Harris 1952).

In his transplantation experiments, as in the case of the remote control electrical stimulation he had previously devised, Harris's holistic concern with the investigation of a fully integrated range of bodily functions in healthy animals was the distinctive hallmark of his experimental approach and enabled him to identify the overall significance of highly specific local events. An excellent example is the rapid onset of reproductive functions observed in adult hosts with neonatal pituitary tissue (Harris & Jacobsohn 1952). Because at this time the donor pituitary tissue age was still considerably pre-puberal, Harris reasoned that the delay in onset of reproductive function that occurs in normal



**Figure 5.3**

Dual, neural, and endocrine control of lactation. The two postulated neuro-hormonal reflexes underlying the stimulating action of suckling on milk secretion. During pregnancy, the cumulative action of the lactogenic hormone (prolactin) from the anterior pituitary causes a buildup of milk, which is, however, retained in the breast until the stimulus of suckling activates neural pathways. This activation leads to the discharge of posterior pituitary oxytocic hormone, which triggers milk ejection (the "let-down" reflex). (Adapted from Figure 46 in Harris 1955.)

development (i.e. puberty) is due not to an inability of the immature pituitary to respond, but to the time needed for maturation of the CNS (Harris 1955).

#### Hypothalamic Control of the Adrenal Cortex

"It is clear," Harris wrote in his monograph of 1955 (Harris 1955),

that environmental factors exert a profound effect on the secretory activity of the endocrine glands, but the mechanism by which these effects are produced is known only in outline. For example, trauma to a limb may result in a flexor reflex response in that limb, and also lead to a discharge of hormones from the adrenal cortex. Much could be said of the sequence of events starting in trauma and ending in contraction of the flexor muscles, but until recently very little could be said about the mechanism linking trauma and discharge of adrenal cortical hormones.

The mechanism of the control of adrenocortical secretion has always presented one of the most complex problems for analysis. This is because of the extreme lability of adrenal cortical hormone secretion in response to a wide variety of stressful and other factors, acting both immediately and over the long term, and also because the adrenal cortex can maintain a basal level of secretion in the absence of the pituitary (Vogt 1951).

Anyone attempting today to disentangle the immense number of conflicting papers and the mass of data published at that time (see for instance Selye 1950) might marvel that Harris had the insight and clarity of thought to extract the central fact, which seems so simple to us now with hindsight, that the control of the secretions of the adrenal cortex depends on central nervous integration of stressful and other inputs. As in the case of the anterior pituitary control of reproduction, this control is exerted by modulating the secretion of what were at that time unidentified factors passing through the hypothalamo-hypophyseal portal vessels to regulate the release of ACTH from the anterior pituitary.

In favor of a central nervous control of pituitary ACTH release, Harris quoted the observation (Sayers 1950) that after removal of their adrenals (to avoid the complications due to feedback both from the cortex and the medulla of the adrenal), animals respond to stress by an increased output of ACTH. In their transplantation experiments, Harris & Jacobsohn (1952) found that adrenal atrophy was only avoided if the pituitary grafts were revascularized by the portal vessels of the median eminence. This indicated a role for the hypothalamo-hypophyseal neurovascular axis in the control of the adrenal cortex.

With Jack de Groot, Harris used the lymphopenic response to the release of adrenal glucocorticoids to show that the pituitary release of ACTH could be evoked by remote control electrical stimulation of the posterior hypothalamus (de Groot & Harris 1950), and that the lymphopenic stress response of rabbits to restraint was abolished by hypothalamic lesions, hypophysectomy, or cutting the pituitary stalk under circumstances where regeneration of the portal vessels was prevented by inserting a plate of waxed paper (Fortier et al 1957).

Harris concluded that although the negative feedback of adrenal cortical hormones "sets a . . . base-line level of secretion against the background of which other factors adjust pituitary activity according to the needs of the organism" (Harris 1955), the CNS plays the key role in the response to stressful physical and psychical events in the environment, and it does so by means of factors that are channeled through the hypothalamo-hypophyseal portal vascular system to

control the secretion of ACTH by the pituitary gland. [They also, as we now know, control the circadian sleep/activity rhythm of ACTH (e.g., Moore & Eichler 1972, Raisman & Brown-Grant 1977).]

### Hypothalamic Control of the Thyroid

Harris & Jacobsohn's transplantation experiments (1952) had demonstrated that, as in the case of the gonads, the maintenance of the functional status of the thyroid gland (on histological criteria) also depended on the grafted pituitary tissue becoming revascularized by the hypothalamo-hypophyseal portal vessels.

Extending this work with Keith Brown-Grant, a recent Cambridge medical graduate, and Sy Reichlin, a young American internist from St. Louis, Missouri, Harris used the rate of release of organically bound radioiodine from the thyroid gland (over a period of days) to measure thyroid activity. By this method, they demonstrated that if stalk section is carried out in rabbits, with precautions to prevent portal revascularization, the basal level of thyroid activity decreases (Brown-Grant et al 1954). In addition the acute, stress-induced inhibition of thyroid secretion is abolished.

Remote control electrical stimulation of freely moving rabbits, with the electrode tip in the median eminence, caused a consistent increase in thyroid activity, provided the complicating effects of the associated induced adrenal hypersecretion were eliminated by adrenalectomy (Harris & Woods 1958). A similar pattern of thyroid response could be elicited by a single injection of anterior pituitary thyrotrophic hormone (TSH) (Campbell et al 1960), thus suggesting that electrical stimulation of the median eminence had produced its effect by releasing a TSH-releasing factor that was carried through the portal vessels to the anterior pituitary gland.

### The Mechanism for Neural Control of the Anterior Pituitary

It was because his investigations encompassed multiple aspects of the physiological and behavioral status of the individual animal that Harris was able to formulate a general mechanism for the neural control of the whole endocrine system. He proposed that specific hypothalamic-releasing factors carried through the local portal vascular system could activate the anterior pituitary in situ or a pituitary transplanted into the portal vascular field but that they would not be present at high enough concentrations in peripheral blood to influence anterior pituitary tissue transplanted away from the portal vascular field. The anterior pituitary in effect "amplifies" the effect of local releasing factor signals by secreting pituitary hormones at levels that are effective through the peripheral blood and can

therefore control distant target endocrine organs, which will, as a result, function wherever they are transplanted.

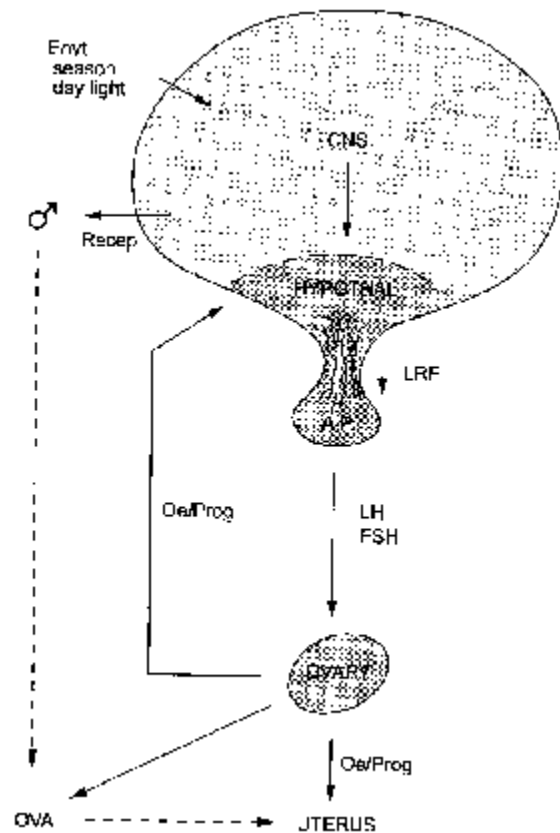
Putting together his own and others' data from localized stimulation and lesion studies, Harris assembled a tentative map of the hypothalamic regions involved in control of the different anterior and posterior pituitary hormones (Harris 1955). A comparable, but less well known map was constructed by Halász and coworkers (Szentágothai et al 1968) on the basis of the novel approach of studying the histological and functional status of anterior pituitary tissue grafted into various positions in the ventro-basal hypothalamus.

### Endocrine Control of Behavior

In 1952, at the invitation of Sir Aubrey Lewis, Harris moved from the Department of Physiology at Cambridge to the specially created Fitzmary Chair of Physiology in the Institute of Psychiatry at the University of London. This provided an intellectual environment where he was to develop his lasting interest in the psychiatric aspects of clinical endocrine disorders, and to set up experiments investigating how the effect of hormones on the brain influences behavior.

Up to now Harris's published work had concentrated on the way in which the hypothalamo-pituitary axis controls the peripheral target organs: gonads, adrenal, thyroid. For the integration of the system into a full adaptive behavior pattern, the organism needs not only to regulate glandular secretion (and ovulation), but also to display appropriate behavior patterns. In reproduction, for example, ovulation must be coordinated with behavioral receptivity (estrus), mating must be followed by preparation of the uterine endometrium for implantation, and pregnancy and parturition must be accompanied by the induction of maternal behavior such as nest-building and nursing. This integration is accomplished by a return feedback of target gland hormones that inform the CNS of the state of readiness of the endocrine system (figure 5.4).

In a preliminary study at Cambridge, Harris had attempted to elicit sexual behavior by implanting estrogens directly into the brain of ovariectomized rabbits, but the appearance of positive responses in the controls had prevented firm conclusions (reviewed in Harris 1964). Now, in collaboration with Patricia Scott and a young psychiatrist, Richard Michael, Harris demonstrated that the implantation of crystals of stilboestrol esters in the hypothalamus of ovariectomized cats elicited full mating behavior even though the genitalia remained atrophic (Harris & Michael 1964). These animals were, therefore, estrous from the point of view of the CNS, and anestrus from the point of view of the endocrine periphery. They established the concept that



**Figure 5.4**

Control of reproduction. A schematic representation showing the pivotal role of the hypothalamo-hypophyseal link in the integration of the neural and endocrine systems to produce a coordinated response to the environmental stimuli that signal the onset of breeding. The maturation of the gonads and ovulation are stimulated by environmental inputs such as the seasonal increase in day length. This input is translated by the CNS into stimulation of the hypothalamic neurons, leading to an increased secretion of luteinizing hormone-releasing factor (LRF), which is carried to the anterior pituitary (AP), where it elevates the secretion of the gonadotrophic hormones LH and FSH. Under their influence the ova mature and are released, and the ovary increases the secretion of estrogen and progesterone (Oe/Prog) to prepare the uterine endometrium for implantation. At the same time, the ovarian hormones also feed back into the CNS to trigger receptivity to the male. This system ensures that mating occurs when ripe ova are available, and fertilization is coordinated with the endometrial changes needed for implantation. [Outline adapted from Harris & Naftolin (1970).]

the brain was a target for the specific feedback action of gonadal steroids to elicit mating behavior.

### The Hypothalamo-Hypophyseal Axis and Seasonal Onset of Reproduction in the Ferret

While Marshall's interest had focused on the effect of seasonal and other exteroceptive factors on reproduction, Harris's work so far had involved the use of artificial factors, such as localized electrical stimulation, as laboratory models for the natural control of the release of anterior pituitary hormones. With his graduate

student Bernard Donovan, Harris now turned his attention to the analysis of the mechanism of action of a natural, exteroceptive factor—the induction of estrus in sexually quiescent (winter) ferrets (Donovan & Harris 1954). For this they used Marshall's demonstration that precocious estrus could be induced in the laboratory by extending the daily animal house lights-on period to simulate the natural seasonal lighting effect (Marshall 1940).

Donovan & Harris (1954) reported that, in the ferret under conditions where regeneration of the portal vessels was prevented by the insertion of a waxed paper plate between the base of the brain and the pituitary gland, section of the pituitary stalk prevented the light-induced onset of estrus. This observation brought them into direct conflict with Solly (later Lord) Zuckerman, one of the most politically powerful UK scientists of the day, and a person who might well have impeded Harris's coveted election to the Royal Society. Based on two cases, Thomson & Zuckerman (1953) had made the claim that pituitary stalk section did not prevent light-accelerated induction of estrus in the ferret. One can therefore imagine with what feelings Harris insisted, as politely as he could (see p. 88 in Harris 1955), that Thomson and Zuckerman had been misled by the fact that the quality of their india ink perfusion technique was inadequate to exclude regeneration of the portal vessels.

Harris was elected to the Royal Society in 1953, and in a review in *Nature* of Harris's seminal monograph of 1955, Zuckerman contented himself with describing Harris's theory as “an edifice of speculation . . . that has been erected because of an urge to explain the incomprehensible” (Zuckerman 1956). What would finally vindicate Harris's position would be the isolation of his proposed hypothalamic factors from extracts of the median eminence, the demonstration of their regulated secretion into the portal vessels, their functional effects on the anterior pituitary gland, and their molecular nature. But these were to be a long time coming. In fact it was to be 14 years from the publication of his 1955 monograph before the first of Harris's postulated hypothalamic hormones (the TSH-releasing factor, TRF) was finally identified (see below).

### The First Steps in the Search for the Hypothalamic-Releasing Factors

Harris's earliest attempts to identify the putative releasing factors were based on screening the then known neurotransmitters and neuromodulators—adrenergic [as originally suggested by Friedgood (1950)], cholinergic, or histaminergic—for effects on the control of anterior pituitary gonadotrophic or adrenocorticotrophic

secretion (Harris 1955, Donovan & Harris 1956). It was well established that central cholinergic and adrenergic blockers inhibited ovulation (Sawyer et al 1949). Experimentally, these substances were valuable in providing ovulation blockers that could be used to dissect the time course of the events induced by an ovulatory stimulus (e.g., Everett & Sawyer 1950, Nikitovitch-Winer 1962, Everett 1964). Harris assumed, correctly, that these blockers were not acting as direct antagonists of the putative releasing factors, but were acting upstream, i.e. by modulating neural activity in the hypothalamus.

The germ of the idea that the releasing factors might be peptides perhaps came to Harris from his earlier experiments on the posterior pituitary at Cambridge (Harris 1947, Cross & Harris 1952). In 1949, Bargmann had used the Gomori stain to demonstrate that the posterior pituitary is directly innervated by perivascular terminals of the axons of the neurosecretory neurons lying in the magnocellular hypothalamic nuclei (Bargmann 1949). The Gomori-stained substance was later shown to be the precursor of the posterior pituitary polypeptide hormones (Livett et al 1971), which are synthesized in the supraoptic and paraventricular neurons and carried down along their axons (which form the neural component of the pituitary stalk) to their release sites on the outer layer of the double basement membrane surrounding the fenestrated capillaries of the posterior pituitary gland.

In 1955, Du Vigneaud had received the Nobel Prize for the elucidation of the nonapeptide structures of the posterior pituitary hormones vasopressin and oxytocin and the synthesis of vasopressin (references in Harris 1955). A similar recognition might well greet the discovery of the structure of the releasing factors that Harris had postulated to control the secretion of the anterior pituitary hormones.

The pituitary nonapeptides of terrestrial animals arise by evolutionary divergence from a single fish nonapeptide gene (Sawyer 1964). Fish have a median eminence, but no separate posterior pituitary. As the enlarged posterior pituitary of terrestrial animals develops, it becomes separated from the rest of the median eminence by the development of the pituitary stalk. Harris suggested that “secretions of the median eminence are perhaps chemically related to the posterior pituitary polypeptides (which may be evolved from the median eminence principles)” (Harris & Campbell 1966). For the different anterior pituitary releasing factors, Harris envisaged a spectrum of related polypeptides, possibly with overlapping physiological activities.

In 1955, both Guillemin (Guillemin & Rosenberg 1955) and Schally (Saffran et al 1955) had started the search for ACTH-releasing activity in hypothalamic

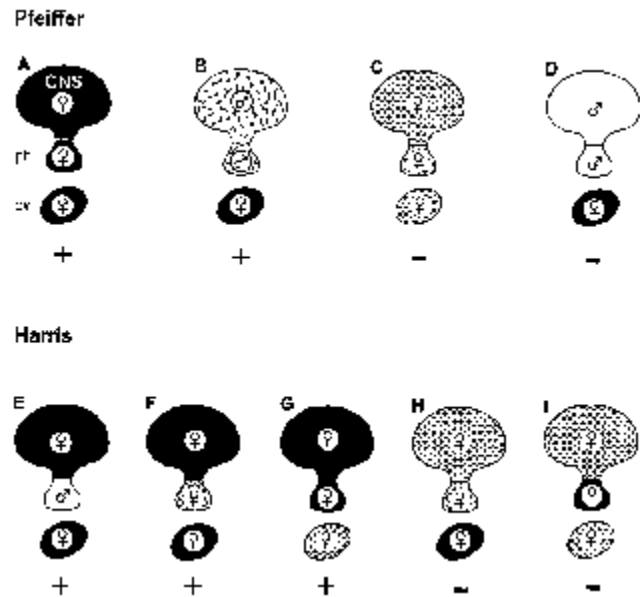
extracts. In 1961, Campbell, Feuer, Garcia, and Harris reported the preparation of a crude acidic (polypeptide) extract of median eminence (Campbell et al 1961). In over 200 rabbits, they applied a meticulous technique based on very slow infusion over 2 h (to simulate the natural time course) through chronically fixed intrapituitary cannulae whose position was monitored by X ray. The LH-releasing activity of the extracts was confirmed by induction of ovulation in these rabbits (Campbell et al 1964), as well as in rats in which spontaneous ovulation had been blocked by Nembutal, a drug acting on the CNS (Nikitovitch-Winer 1962). This finding indicated that the effect was exerted at the level of the pituitary gland, i.e., downstream from the hypothalamus. The intrapituitary route of administration was considerably more effective than intravenous injection into the peripheral blood stream. This is consistent with the view that the extracts contained factors normally produced in such minute amounts that they had to be sequestered into the localized hypothalamo-hypophyseal portal mini-circulation in order to achieve high enough concentrations to affect the anterior pituitary.

Starting a year earlier, McCann et al (1960) had also prepared median eminence extracts, and reported stimulation of pituitary LH secretion, as demonstrated by the Parlow bioassay of ovarian ascorbic acid depletion in the rat (Parlow 1958), and similar results were reported by Courrier et al (1961). What Wade (1981) called the "Nobel Duel" had begun, but for different reasons, neither Harris nor McCann were to be among the finalists.

### Sexual Differentiation of the Brain

Harris & Michael's work (1964) on the induction of cat estrous behavior by the action of gonadal steroid hormones on the adult brain was the prelude to a remarkable series of studies showing the dramatic and permanent effects produced by these same steroids on the developing brain of the rat (figure 5.5).

That the control mechanism for ovulation is present in female animals but not in males was well known: An ovary transplanted into a gonadectomized adult female is able to ovulate (Marshall & Jolly 1907), whereas an ovary transplanted into a gonadectomized adult male is not (figure 5.5D) (Goodman 1934). These observations apply to adult, sexually mature animals. For some time, however, there had been an interest in how the different male and female sexual patterns developed and what controlled them. In the second edition of his classical textbook, *The Physiology of Reproduction*, Marshall (1922) proposed the issue in terms that continue to resonate in our understanding of sexuality to the present day:



**Figure 5.5**

Sexual differentiation. (*Upper row*) Ovulation (+) occurs in normal genetic female rats (A), but not (–) in ovaries (ov) transplanted into normal genetic males (D). Pfeiffer (1936) demonstrated that the later development of ovulation in the adult is prevented by transplantation of a testis into a newborn female (C), and that castration of the newborn male allows the development of a mechanism by which the adult can induce ovulation in a transplanted ovary (B). From this experiment it remained possible that sexual differentiation resided in the pituitary (pit), or in the CNS. (*Lower row*) The work of Harris & Jacobsohn (1952), Harris & Levine (1965), and others (for a summary see Harris & Campbell 1966) demonstrated that sexual differentiation resided in the CNS and not in the pituitary. They obtained androgen-sterilized females by a single neonatal injection of testosterone. They showed that a normal female will ovulate if her pituitary is replaced by that of a male (E) or an androgen-sterilized female (F). In addition, the brain/pituitary axis of an ovariectomized normal female host is able to induce ovulation in an anovulatory ovary transplanted from an androgen-sterilized female (G). Thus, both the pituitary and the ovary of androgen-sterilized females can maintain cyclic function. Conversely, a normal female ovary will not ovulate if transplanted into an androgen-sterilized female (H), and replacement of the androgensterilized pituitary with a normal female pituitary transplanted into an androgen-sterilized female (I) will not induce ovulation. This combination of experiments proves conclusively that sexual differentiation is a property of the CNS. Black, normal female; grey, androgen-sterilized female; white, normal male, speckled, neonatally castrated male. ♀ indicates genetic females; ♂ indicates genetic males. +, ovulation; –, ovulation does not occur.

While the chromosome constitution may determine sex at fertilisation, in some instances this is clearly overridden during subsequent development, and this results in the production of "somatic" males or masculinised females. The mechanism by which the female is transformed is one which acts through the internal secretion of the gonads.

If it be true that all individuals are potentially bisexual, the dominance of one set of sexual characters over the other may be determined in some cases at an early stage of development in response to a stimulus which may be either internal or external. The observations upon animals of many different kinds point even to the possibility that sex may be reversed after it has once been established.

When once we admit the existence of latent sexual characters in individuals in which the characters of one sex are dominant, and that under certain circumstances those of the latent sex can develop at the expense of the dominant ones, we are compelled to acknowledge also that the sex of the future individual is not always predetermined in the gametes or even in the fertilised ovum, but may be called into being at a later stage in life.

But although Marshall had raised the possibility of the hormonal control of sexual differentiation, little was known about the mechanism by which it might be brought about in normal development, and few would have suspected that the crucial target for sexual differentiation would be found to be the brain.

#### Pfeiffer's Experiments

In 1936, Pfeiffer showed that neonatal ovariectomy did not prevent the female rat from developing into an adult capable of maintaining cyclic ovulation in a transplanted ovary (Pfeiffer 1936). On the other hand, the implantation of testes into female rats at birth caused a permanent loss of the ability to ovulate (figure 5.5C). Thus in the genetic female, the development of the ability to ovulate does not require that an ovary be present during development, but the presence of a testis can prevent it.

Pfeiffer also showed that genetic males castrated at birth develop into adults in which the presence of an intact ovulatory control mechanism could be revealed by transplantation of an ovary (figure 5.5B). That this was a permissive effect due to absence of the testicular secretion was demonstrated by the fact that testes immediately transplanted back into the neck of a neonatally castrated male were sufficient to suppress the development of the ovulatory mechanism.

Pfeiffer concluded that both females and males have the potential to develop a female-type cyclic ovulatory mechanism. If gonadectomy was performed at birth in either genetic sex, the resultant adult developed a female-type ovulatory mechanism. Testes present in rats of either genetic sex during the neonatal period induce a male-type noncyclic mechanism.

One possible interpretation of these observations was that the anterior pituitary gland was the target for sexual differentiation. Subsequently, however, the work of Harris and others established a compelling case that the mechanism for triggering ovulation resides in the CNS, e.g., stimulation of the hypothalamus induces ovulation (see above), hypothalamic lesions, cutting the pituitary stalk, or transplantation of the pituitary to a site away from the portal vascular field, all prevent ovulation, and drugs acting on the CNS block ovulation (reviewed in Harris & Campbell 1966). This raised the possibility that sexual differentiation also might oc-

cur not at the pituitary level, but in the CNS (Everett et al 1949).

#### Harris's Experiments

In their transplantation experiments published in 1952, Harris & Jacobsohn (1952) had found that not only neonatal pituitaries of either sex, but also adult male pituitaries can support estrous cycles, mating, pregnancy, and milk production when transplanted into hypophysectomized adult female hosts (figure 5.5E). This established that the pituitary was not sexually differentiated, and further favored the view that the sexually differentiated cyclic ovulatory mechanism was located in the CNS.

By 1960, it was known that the sterilizing effect of a testis transplanted into neonatal females could be obtained more conveniently by a single injection of the long-lasting testosterone propionate ester, and that the critical period for obtaining this effect was limited to the first week after birth (for references, see Barraclough 1961). Using this method, Harris & Seymour Levine (1962, 1965) reported a series of studies that conclusively showed that the sexually differentiated mechanism lay in the CNS.

With Harris's characteristic thoroughness, they analyzed the mechanism of the anovulatory defect induced in adult female rats by administration of testosterone propionate during the critical postnatal period. They noted that the ovaries of these adult genetic females were able to maintain a state of persistent vaginal cornification (which requires the secretion of estrogen) but that they did not ovulate. High doses of exogenous estrogen caused ovarian atrophy, and ovariectomy caused castration cells to appear in the anterior pituitary. These observations showed that the negative feedback of ovarian steroids on the anterior pituitary is functionally intact in androgen-sterilized females. However, the positive feedback of estrogen—i.e., induction of the ovulatory surge of LH secretion—is absent. The animals did not show female behavior (enticing and lordosis) under conventional testing conditions with a normal male, and they expressed male behavior (mounting and intromission) when tested with a receptive female.

That the failure of ovulation in androgen-sterilized females is not due to an impairment of the ovaries was confirmed by showing that when the anovulatory ovaries of an androgen-sterilized female were transplanted under the kidney capsule or [using the technique of Goodman (1934)] into the anterior chamber of the eye in an ovariectomized normal adult female rat (figure 5.5G), they ovulated and maintained 4- or 5-day vaginal cycles in the host (Harris 1964, 1970; Harris & Levine 1965; Harris & Campbell 1966). Therefore the

ovaries had retained the ability to ovulate when stimulated by appropriate pituitary gonadotrophin secretion. On the other hand, normal ovaries transplanted into the anterior chamber of the eye of androgen-sterilized rats did not ovulate (figure 5.5H) (Harris 1964), and normal pituitaries transplanted into hypophysectomized androgen-sterilized rats were not able to reverse the anovulatory status of the hosts (figure 5.5I) (Adams Smith & Peng 1966).

When transplanted into the sella turcica (Segal & Johnson 1959) or the subarachnoid space of the median eminence region (Adams Smith & Peng 1966) of hypophysectomized normal females, the pituitaries from androgen-sterilized rats (as with male pituitary tissue) (see Harris & Jacobsohn 1952, above) could maintain ovulatory cycles, mating, pregnancy, and parturition (figure 5.5F). Therefore the pituitaries of androgen-sterilized rats remained able to drive cyclic ovulatory function, provided they had access to a competent hypothalamic mechanism. From this, Harris concluded that neonatal androgen sterilization was due to an effect, not on the pituitary or the ovary, but upstream of the pituitary, i.e., on the CNS.

In 1962, Harris moved from the Institute of Psychiatry to succeed Sir Wilfrid Le Gros Clark as Dr. Lee's Professor of Human Anatomy at Oxford, where he also set up the Medical Research Council Neuroendocrinology Unit. Here he extended his studies on sexual differentiation to the feminization of neonatally castrated genetic male rats (Harris 1964). Following Pfeiffer's observation of ovulation in a transplanted ovary (1936), Harris wished to know whether these males (figure 5.5B) could actually maintain regular cyclic ovulation with the same periodicity as normal females. Yazaki (1960) had shown that such males transplanted with ovaries and a vagina showed 5-day cyclic vaginal changes and cyclic changes in running activity. Harris now confirmed these results for cyclicality in a transplanted vagina and for running activity, and by direct examination of ovaries transplanted in the anterior chamber of the eye, he was also able to observe at least the first two cycles of ovulation usually had 5 days duration. With appropriate hormone priming, the neonatally castrated males were bisexual, i.e. they showed both male and female behavior patterns. Subsequent work has confirmed the view, originally put forward by Marshall (see above), that the neural circuitry for both male and female sexual behavior is present in rats of both sexes (e.g., Beach 1971).

Thus, regardless of the genetic sex, the neural circuitry present at birth in both male and female rats can lead to development of a cyclic, female type of ovulatory control mechanism in the adult CNS. For both sexes, the basic pattern of development of this mechanism is female. In the normal life history of the

male, the development of the cyclic ovulatory mechanism is prevented by his own testicular secretions during the critical neonatal period. The female is not exposed to adequate levels of steroids during the critical neonatal period, so her CNS develops the basic cyclic ovulatory generator. However, when exogenous androgen or other steroids (see references in Harris & Campbell 1966) are administered to the newborn female, the effect is the same as that of the endogenous testicular steroids of the male—the development of the adult CNS cyclic ovulatory pulse generator is prevented.

As Harris pointed out (1970), this sexual differentiation of the CNS is part of a wider picture. Thus, at an earlier stage of development, gonadal hormones also control the differentiation of the genital tract. Secretions from the embryonic testis cause regression of the female-type Mullerian duct system (Behringer 1995) and induce development of the male-type Wolffian duct system (Jost et al 1972). During this somatic and the later CNS development, the basic default pattern appears to be female. The switch to male-type absence of the CNS ovulatory pulse-generating mechanism is induced in normal development by exposure to endogenous testicular steroid secretions, and experimentally by administration of exogenous steroids. The common factor was later shown to be the conversion of these steroids to estrogens by a local aromatase mechanism that is present in the hypothalamus and limbic system during the period of sexual differentiation in the rat (Reddy et al 1974, Naftolin et al 1975).

#### **Anatomical Evidence for Sexual Differentiation of the Rat Preoptic Area**

Although Harris's work had established that the brain was sexually differentiated, there was no indication of whether this effect was localized, and if it was, to which region. The prime candidate for such localization would be the part(s) of the brain involved in generating the sexually dimorphic functions, of which the most obvious is ovulation. An accumulation of data from stimulation and lesion experiments (reviewed in Harris & Campbell 1966) had shown that the central ovulatory mechanism in the rat depends on the integrity of the preoptic or suprachiasmatic area. Thus the ovulatory signal from the preoptic area can be blocked by exposure to constant light or by administration of Nembutal at pro-estrus, and in these situations, ovulation can be induced by mating or by electrical stimulation of the preoptic area. If the preoptic area is destroyed, electrical stimulation does not induce ovulation. In the same way, electrical stimulation of the preoptic area does not induce ovulation in the androgen-sterilized female (Barraclough & Gorski 1961). This

suggests that the effect of the neonatal testosterone on development is similar to that of a preoptic lesion in the adult, viz, it irreversibly abrogates an ovulatory trigger mechanism located in the preoptic area.

In his 1966 review, Harris noted: "It would be of much interest to make detailed studies of this region with the electron microscope to see if any difference can be detected between the normal female on the one hand, and the androgen-sterilized female or normal male on the other" (Harris & Campbell 1966).

By chance, when Harris arrived at the Department of Human Anatomy at Oxford, Pauline Field and I were developing an electron microscopic sampling procedure to count the number of synapses in specific projection pathways in the CNS. We had used this approach to demonstrate the reinnervation (plasticity) of denervated postsynaptic sites in the adult septal nuclei (Raisman 1969), and we were currently examining the recently described projection of the amygdala to the hypothalamus (Raisman 1970) and comparing it with the amygdaloid projection to the preoptic area.

To our surprise, a comparison of individual rats consistently showed a bimodal distribution in the number of a particular category of spine synapses in that part of the preoptic area receiving amygdaloid input. When we showed this puzzling observation to Keith Brown-Grant, his immediate question was something at that time largely disregarded by neuroanatomists: "What was the sex of the animals?" Going back to the records, we discovered that indeed the animals with the fewest specific spine synapses were all males. This was the first demonstration of sexual dimorphism in numbers of synapses in the brain (Raisman & Field 1971). The possible relationship to the ovulatory mechanism was further supported by Velasco & Taleisnik's demonstration (1969) that stimulation of the amygdala induced ovulation and an increased pituitary LH secretion in female rats (but no increase in LH in castrated estrogen-primed male rats), and that these responses were blocked by lesions of the pathway leading from the amygdala to the preoptic area.

We therefore set up a developmental series of rats of both sexes. The females were treated with a single 1.25-mg injection of testosterone propionate (Barraclough 1961) either before or after the critical period for sexual differentiation. With Harris's direct instruction, and the gift of his own personal microsurgical instruments, the males were castrated either on the first day of life or after one week. The results were counted "blind." They showed that the anatomical sexual dimorphism in the preoptic area was differentiated in exactly the same way as Pfeiffer and Harris had described for the ovulatory mechanism, i.e. it depended not on the genetic sex of the animal but upon the presence or absence of gonadal steroid hormones during the critical period of

the first week of postnatal life in the rat (Raisman & Field 1973).

#### **Measurement of Luteinizing Hormone-Releasing Factor in Portal Blood**

But now the wheel of Harris's life was coming full circle. In the introduction to his 1955 monograph, Harris had written: "The ultimate test by which the number of substances secreted by a given endocrine gland will be decided is the qualitative and quantitative analysis of the hormones in the venous blood from the gland." It was as though, two decades earlier, he had been given an intimation of the end point of his own life's work.

In collaboration with George Fink, a Nuffield Demonstrator from Monash University, Australia, Harris applied the method for collection of rat portal vessel blood described by Curtis Worthington (1966). Using the Parlow ovarian ascorbic acid depletion bioassay for LH, they were able to detect LH-releasing activity [luteinizing hormone-releasing factor (LRF), now called GnRH; see below] in samples (after inactivation of any LH in the sample). But they failed to detect the expected rise in LRF during the pre-ovulatory critical period of pro-estrus (Fink & Harris 1970). With hindsight this was due to suppression of the pre-ovulatory surge by the anesthetic used. Electrical stimulation of the hypothalamus at a level sufficient to overcome Nembutal block of ovulation did cause a surge in LRF activity in the portal blood in all phases of the rat cycle except estrus (Harris & Ruf 1970, Fink & Sheward 1989).

George Fink persevered in improving the technique of portal vessel blood collection (Fink & Sheward 1989) and finally discovered a method of steroid-based anesthesia that showed unequivocally that the level of immunoassayable LRF increases abruptly immediately before the pre-ovulatory surge of anterior pituitary LH on the pro-estrous day of the rat cycle (Sarkar et al 1976). Harris's final criterion was thus fulfilled: The time of appearance of the active agent in the venous effluent from the hypothalamic gland was correlated with, and necessary for, its proposed physiological function.

#### **The Race for the Identification of the Ovulatory Signal**

But Harris had set himself yet another goal that, had he been successful, would have given him the molecular nature of the ovulatory signal released from the hypothalamus. As it was, other laboratories, building on the knowledge Harris had uncovered, but with greater resources and greater molecular expertise, were already moving ahead of him.

Around the world now, the search for the hypothalamic hormones was accelerating, and other groups were using techniques less physiological but far more rapid than those of Harris's induction of ovulation in rats or rabbits. Some years earlier, two papers (Guillemin & Rosenberg 1955, Saffran et al 1955) had reported tissue coculture assays that showed that hypothalamic tissue could induce the release of ACTH from pituitary tissue. In 1969, the groups of Schally and Guillemin, both of whom had previously been involved in a long and as yet unsuccessful search for the identity of the ACTH-releasing factor (CRF), independently worked out that the TSH-releasing factor (TRF) in hypothalamic extracts was the tripeptide pyro-glutamine-histidine-proline amide (Burgus et al 1969, Folkers et al 1969).

In the quest for LRF, Harris's main competitors were the groups of Don McCann in Texas (Ramirez et al 1964), Andrew Schally in New Orleans, and Roger Guillemin at the Salk Institute in San Diego. In 1964, Schally & Bowers, using both in vivo and in vitro assays, proposed a tentative 11-amino acid residue composition for bovine LRF peptide (Schally & Bowers, 1964), and the next year Guillemin proposed 9-amino acid residues for ovine LRF (Guillemin 1977a).

Like Dora Jacobsohn, Marthe Vogt was a refugee from Nazi Germany and a great admirer of Harris, both as a scientist and a hater of injustice. In the Royal Society Biographical Memoir of Geoffrey Harris, she wrote:

The race for the isolation of the luteinizing hormone releasing factor, LRF, had started in the USA with such a drive and with such vast financial resources that, seen retrogradely, there was little chance for Harris of being the first to arrive. Harris was too careful a worker to win a battle in which speed was decisive.

Compared with the highly directed research teams of Schally and Guillemin, with their major support from the US National Institutes of Health, Harris's style of associating with collaborators with independent scientific programs, and the terms of his arrangement with the UK Medical Research Council, did not provide him with the same scale of resources to assign to the purchase and large-scale purification of median eminence extracts. The meticulous surgical precision that had been such a strength of Harris's electrical stimulation work was a disadvantage when he attempted to use the all-or-none rabbit ovulatory response to intrapituitary infusion as an assay in the purification of the hypothalamic ovulatory hormone (Campbell et al 1964). His competitors were able to process material much faster using the quantitative Parlow ovarian ascorbic acid depletion test (Parlow 1958) and, sub-

sequently, radioimmunoassay (Niswender et al 1968, Harris et al 1970, Harris 1972).

For the chemistry, Harris had the assistance of Harry Gregory, a distinguished ICI scientist who had previously solved the structure of gastrin but who was only permitted to spend part of his effort in the purification of LRF. By 1968, in collaboration with May Reed and Harry Charlton, they used 50,000 hypothalami to obtain a purified preparation of a putative LRF polypeptide, with a molecular weight of around 1000 to 1500, indicating between 9 and 13 amino acids (Gregory et al 1968). Harris was in sight of the goal.

In 1971, Naftolin, Harris, and Bobrow used the purest preparation of this putative LRF diagnostically to show that a pituitary LH response could be elicited in two human patients with Kallmann's syndrome (familial hypogonadism with anosmia) after priming the pituitary with an estrogen agonist (Naftolin et al 1971). This finding indicated that the pituitary was competent to respond to appropriate stimuli and therefore that the defect in the disease lay in the CNS. [It is now known to be due to failure of migration of the GnRH neurons (Schwanzel-Fukuda et al 1989).] "This work," Naftolin commented, "shows the physician in Harris." It foreshadowed the future diagnostic use of GnRH (see below).

But by 1971, the competition was over. In that year, Schally's group used the extracts of over a million pig hypothalami to purify a decapeptide with LRF activity, determined its amino acid sequence to be pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH<sub>2</sub> (Matsuo et al 1971b), synthesized a molecule with the predicted structure, and demonstrated that it had biological activity indistinguishable from the natural factor and was effective in inducing ovulation at a threshold of 0.5 ng in rats (Matsuo et al 1971a). Their extracts induced a rapid rise in LH in human pre- and post-menopausal females and males (Kastin et al 1970). They showed that the same hypothalamic molecule controlled the anterior pituitary secretion of both LH- and follicle-stimulating hormone (Schally et al 1971), and they renamed it gonadotrophin hormone-releasing hormone (GnRH), making it, as Wade (1981) said, "the hormone that governed the breeding behavior of pigs, and probably of man and all other mammals as well."

In the same year, Schally's group reported a tripeptide sequence for melanocyte-inhibiting factor (which regulates the secretion of the pars intermedia of the pituitary) (Nair et al 1971), and in 1973, Guillemin's team published the structure of somatostatin, the hypothalamic hormone that regulates pituitary growth hormone secretion (Brazeau et al 1973).

At last the existence of Harris's long-postulated hypophysiotrophic hormones was being proved, but

all that now becomes part of another story. Harris's is over.

In his 1971 Henry Dale Lecture, it was typical of Harris that he should have written: "The studies... especially of Schally and his co-workers in New Orleans, will undoubtedly stand as a milestone in the history of endocrinology."

The lecture was published the year after Harris's death (Harris 1972). For the elucidation of the nature of the chemical messages from the hypothalamus to the pituitary, Schally and Guillemin shared the Nobel Prize of 1977 with Rosalyn Yalow, who received it for her part in developing the technique of radioimmunoassay (see Fink 1977, Meites 1977).

### Conclusion

Looking back over the 25 years since his death, the work of Geoffrey Harris towers over the surrounding landscape like a solitary mountain peak, standing clear of all around. In the First Geoffrey Harris Memorial Lecture of the International Society for Neuroendocrinology (Sawyer 1975), Charles Sawyer wrote,

Harris was not the first to suggest that the adenohypophysis might be controlled by a humoral mechanism involving the hypophysial portal system, but it was the force of his intellect, personality and multifaceted research approach to the problem in the late 1940's and early '50's which really established the neurovascular concept.

Harris's professional life started in the Cambridge Physiology Department, where the Nobel Prize winners Hodgkin, Huxley, and Adrian were heroes of the study of electrical transmission in the nervous system. Against the background of their achievements, Harris had to fight hard to establish the credibility of a control mechanism that required humoral transfer of neural information. Not only in Cambridge, but throughout the world at that time, the chemical theory of neurotransmission was still hotly debated for the peripheral nervous system, let alone for the CNS (e.g. Feldberg 1945).

Harris's contemporaries were comfortable with the concept of the brain as the organ of thought, perception, memory, decision, and movement, but less so with the idea that the brain is also a gland. For the 14 years between his exchange with Zuckerman in 1955 and the demonstration of TRF in 1969, Harris defended his theory of the existence of specific hypothalamo-hypophysial hormones when no such hormone had yet been identified. "What Guillemin and Schally did," Wade wrote in *The Nobel Duel* (1981), "was to look for the entities Harris described in the places where he said they would be found."

Following Marshall's lead, Harris's work had consistently moved the endocrine control mechanism

"upwards" from the early days of what he used disparagingly to call "pelvic endocrinology." His work also effectively disposed of the idea of the pituitary as the "conductor of the endocrine orchestra" by showing that the control mechanism actually lay in the brain itself. This was perhaps most unexpected for the demonstration of sexual differentiation.

Harris was the founder of the science of neuroendocrinology. As Yasumasu Arai put it: "Harris put the brain into the endocrine system." He also put the endocrine system into the brain. Subsequent research has shown that neuropeptides of major functional importance are present in all parts of the central and peripheral nervous system (Guillemin 1977b), far beyond the hypothalamus. Hypothalamic peptides such as LRF (Moss & McCann 1973) and CRF (Vaughan et al 1995) have major effects on brain as well as on pituitary tissue.

Harris's work also led to practical advances. GnRH came to be used for diagnostic purposes and therapeutically, not only in its primary, pulsatile role as a means to increase fertility (Mason et al 1984), but more often as a chronic pituitary suppressant (chemical hypophysectomy) in conditions such as prostatic carcinoma, precocious puberty, and endometriosis (Belchetz et al 1978, Crowley et al 1981, Walker et al 1983). But to pick out subsequent developments in the use of individual releasing factors would be to obscure Harris's major intellectual contribution to our present way of thinking, rather as if one were to ask of Darwin's theory: To what specific practical developments has the concept of evolution led?

Extending over nearly half a century, Harris's work forms a bridge from an earlier era. The time seems remote when Marshall could write: "Lord Latymer states that deer have been imported into New Zealand from many sources besides Scotland... but that now, wherever they come from, they all begin roaring about the third week in March" (Marshall 1936).

On the wall of the professorial study in Oxford, hung a framed black and white photograph of a nineteenth century gentleman in a suit of a continental cut, white shirt, and a cravat. Sometimes Harris would point to the photograph and ask: "Do you know who that is?" From his seated position, the respectful visitor would be unable to read the signature crossing the lower corner of the portrait and would need to be told that it was Claude Bernard, the eminent French physiologist, famous for his postulate: "The constancy of the internal milieu is the condition of free life." The ability to colonize widely different and changing external environments requires that the organism, like a high altitude airplane, be provided with a series of fail-safe control mechanisms to shield its internal environment from changes outside. Claude Bernard's concept of

homeostasis was central to the science of endocrinology, in which the internal secretions are regulated by feedback from circulating hormones. Harris set this in a wider context. He identified the hypothalamo-hypophyseal system as the final common pathway for the integrated brain-endocrine response to environmental inputs (figure 5.4).

The concept of the hypothalamo-hypophyseal system has implications far beyond the laboratory. In the pigeon, the sight of another bird or even the reflected image in a mirror is sufficient to induce ovulation (Matthews 1939). The secretion of LRF into the portal vessels is the mechanism by which seasonal changes in day length in ferrets, diurnal lighting rhythms in rats, overcrowding or lack of food in mice, mating in rabbits, or the presence of a mate or eggs in birds regulate the balance between the positive forces of procreation and the negative, or protective, forces of aggression. During the life cycle of each individual, the hypothalamo-hypophyseal axis is the route through which the developing brain signals the onset of the reproductive period (puberty). In the adult, it carries the messages by which trauma, stress, or disturbance of biorhythms affect the reproductive cycle (as in the human menstrual cycle). Through it the aging brain signals the cessation of the reproductive period (menopause). The brain-pituitary axis offers a potential route for planned human population control by interventions to reduce or to increase fertility. It will be a key player in the human struggle to adapt to the ever-increasing world shortage of food and space.

Our understanding of these vital neural mechanisms that are so important for our future is still in its infancy. The events Harris described were cyclic—the cycles of birth and growth, reproduction and death, and the passing of the generations. The combined cycles of individuals merge into those of populations and interact with the external cycles of the days and months, seasons and years.

Great research not only answers questions of its generation, but raises those of the next. One of the great mysteries is how the neural mechanism achieves its coordination of these internal and external cycles, so essential to the survival of the species. At a very basic level, how are signals from the circadian clock in the suprachiasmatic nucleus transmitted to the ovulatory center? How does the hypothalamus of a rat remember it has mated, and maintain ten days of pseudopregnancy? Harris himself started the search for evidence as to whether the human male brain can maintain a monthly cycle of gonadotrophin release comparable to the female. Buried in our brains, and conserved through millennia of evolution, how far do these hidden biorhythms, beyond our conscious control, govern our lives?

Harris was an extremely determined scientist. He had a robust, outgoing character that could inspire colleagues. The list of the names of his students and co-workers forms a roll call of those who led and were to lead the field of neuroendocrinology. His writing was clear and easy to understand. His conclusions were always careful, at times cautious, and he was always aware of the human dimension of his research, the clinical aspects of neuroendocrine malfunctions, and the potential applications of his findings to them.

Harris's scientific life began with a question—how to explain the seasonal effects described by Marshall. Forty-five years later, his final message was also a question: "What benefits will this work and knowledge confer on human welfare?"

### Acknowledgments

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In two recent papers, by Marshall and Verney (1936), and by Harris (1936), the mechanism concerned with ovulation in the rabbit has been fully discussed. In view of this, only a brief summary of the present position will be given here.

Ovulation in the rabbit occurs normally only after some form of sexual excitement. There is much evidence to show that the factors involved are: first, a nervous stimulation from the genital region and perhaps from the cortex, acting on the anterior lobe of the pituitary gland; and secondly, a hormonal factor, the pituitary gland secreting a gonadotropic hormone which affects the ovaries.

The nervous pathway by which the pituitary gland is stimulated has not yet been fully determined. Cajal (1894) was the first to show that the posterior lobe of this gland is in direct nervous connexion with the hypothalamus. More recently, Greving (1925, 1926) and Pines (1925) have shown that the supra-optic and paraventricular nuclei supply the posterior lobe with fine non-medullated nerve fibres passing through the infundibulum. The only known nerve supply of the anterior lobe is from the superior cervical ganglion, through the carotid plexus (Dandy 1913). No fibres have been traced from the pars nervosa to the pars anterior.

The three main hypotheses which have been put forward concerning the pathways of nervous impulses to the anterior lobe of the pituitary are as follows:

1. That the impulses pass along sympathetic fibres in the superior cervical ganglion and the carotid plexus. These are the only nerves which have been found entering this lobe of the gland. That this is not the only path has been shown by Vogt (1931, 1933), Hinsey and Markee (1933), and Brooks (1935), who have all found that rabbits ovulate in a perfectly normal manner after extirpation of the superior cervical ganglia. On the other hand, Haterius (1933) showed that sympathectomized rats failed to become pseudo-pregnant following artificial stimulation, whilst Friedgood and Pincus (1935) managed to obtain ovulation in rabbits after electrical stimulation of the superior cervical ganglia, a

result that Haterius (1934) had previously failed to obtain.

There is therefore evidence that the cervical sympathetic system plays some part in this mechanism, but presumably not a very large part.

2. That there are sympathetic fibres from the central nervous system which supply the pituitary gland, other than those passing through the superior cervical ganglion. Thus Hinsey and Markee (1933) suggest that stimuli may pass to the anterior lobe, via the greater superficial petrosal nerve and the carotid plexus, that is, along paths described by Cobb and Finesinger (1932) and by Chocobski and Penfield (1932).

There appears to have been no experimental evidence put forward for or against this theory.

3. The third possibility is that there occurs a humoral or nervous transmission of stimuli from the posterior to the anterior lobe of the pituitary, the posterior lobe itself being affected by nerve fibres from the hypothalamus. The evidence for this has been mainly of a negative character, concerning the effect of hypothalamic and pituitary-stalk lesions, on the sex cycles of various animals. Camus and Roussy (1920) showed that, in dogs, damage of the hypothalamus performed with a hot needle led to genital atrophy, though the pituitary gland was left intact by the operation. Bailey and Bremer (1921) found that genital atrophy in dogs followed lesions to the tuber cinereum performed by the temporal route so that there was no danger of concurrent damage to the pituitary gland. Smith (1926) found that lesions of the tuber cinereum in rats produced, amongst other effects, genital atrophy. Cushing (1932a), in discussing Smith's results, says that "in all probability this gonadal effect is merely another instance of interference with hypophysial blood supply or of interrupted nerve supply". Richter (1934) cut the stalk, also in rats, and obtained great prolongation of the oestrous cycle.

This evidence certainly seems to suggest that the hypothalamus and stalk of the pituitary gland are concerned with sexual activity, probably as a functional unit, together with the anterior lobe.

The purpose of the present work was to discover whether the hypothalamus and stalk of the pituitary gland played any part in the mechanism underlying ovulation in the rabbit. Three types of experiments were performed to find the effects on the ovaries of; first, lesions of the stalk; secondly, electrical stimulation of the pituitary gland directly; and thirdly, electrical stimulation of the hypothalamus.

### Lesions of the Stalk

The original object of these experiments was to see whether rabbits would still ovulate following copulation, after section or lesions of the stalk.

The operative technique was developed in collaboration with Professor G. T. Popa, for whose invaluable aid and advice I am greatly indebted. A paper will shortly be published describing the surgery in full, so that it will suffice for the present to mention that the temporal route was used, that this included removal of the posterior half of one zygomatic arch and the superior half of the ramus of the mandible. The lesions were made with a small piece of razor blade, which was ground to the required shape and mounted at the end of a fine probe. It should be mentioned that the diaphragma sellae is well developed in the rabbit, so that during suprasellar operations there is no danger of incidental injuries to the pituitary gland.

Many rabbits died in fits within 2–3 days of the operation. It is believed that this was partly due to meningitis (the condition for asepsis being poor), and partly due to injury of the hypothalamus and tuber. (See Bailey and Bremer 1921.)

Eventually six rabbits, four does and two bucks, were obtained, with lesions of varying extent in the stalk. Three showed transient polyuria for a few days following the operation, but a quick recovery to normal occurred in each case.

The following symptoms were the same in all the rabbits. For the period from  $1\frac{1}{2}$  to 5 months following operation the animals were, to outward appearances, normal in every way; they were lively, and plump, and had developed good appetites, but they showed complete lack of sexual interest. These experiments were performed in the early months of the year, December to May, when under normal conditions the does are coming on to heat after the comparatively quiescent period of winter. The two operated bucks, though, also showed this lack of desire, so that it is unlikely that the time of year was the decisive factor.

At the end of this period all the animals commenced to waste. In spite of every possible attention, anorexia developed, the animals becoming more and more emaciated until death occurred from 2 to 7 months after the operation.

On post-mortem, all the animals showed a complete lack of visible fat. The sexual organs were extremely atrophied. The hypothalamus, united to the pituitary gland and the sella turcica, was taken in each case, and serially sectioned. After staining the sections with haematoxylin and eosin, the lesions were verified histologically (figures 6.4 and 6.5, plate 15). In all the animals, it was found that distal to (that is, on the pituitary side of) the lesion the stalk and posterior lobe were shrunken and very cellular (compare figures 6.6 and 6.7, plate 15). Also in two of the glands a large accumulation of colloid was observed in the remains of the posterior lobe (see figure 6.4). Gersh and Tarr (1935) have lately questioned the function of this colloid. They suggest it might be a histological artifact. If this is true, it is difficult to see why the colloid should increase in amount after lesions of the stalk. In the immediate vicinity of the lesion there was also a varying accumulation of small, darkly staining cells, again sharply localized to the distal segment of the stalk. Crowe, Cushing and Homans (1910), and Cushing and Goetsch (1910) also noted these effects after similar experiments. The number of cells in the anterior lobe appeared greatly increased. The individual cells, in this lobe, seemed to have lost most of their cytoplasm and this, together with the fact that the nuclei stained darkly with haematoxylin, gave the general appearance of a collection of small lymphocytes (figures 6.8 and 6.9, plate 15). It was noticed that the anterior lobe was still very vascular.

According to Popa and Fielding (1930), there is no reason why lesions of the stalk should interfere with the systemic circulation of the pituitary gland, the arterial supply coming directly off the internal carotid artery, and a proportion of the venous drainage returning to the cavernous sinus. In this case, it follows that the atrophy of the pituitary noted above is probably due to interference with the nerve supply. It appears, therefore, that lesions of the stalk lead to degeneration of the pituitary gland accompanied by the disappearance of sexual desire. As regards the cause of the degeneration, the results to be given seem in no way adverse to the suggestion just made, so that the degeneration observed may be due to lack of (perhaps, tonic secreto-motor) nervous impulses, rather than, or in addition to, lack of blood supply.

### Stimulation of the Pituitary Gland and Hypothalamus

In developing the technique for these experiments, I owe a deep debt of gratitude to Dr. J. Beattie and Professor W. R. Hess, both for their invaluable advice and for their kindness in permitting me to work for a short time in their laboratories in order to study their techniques.

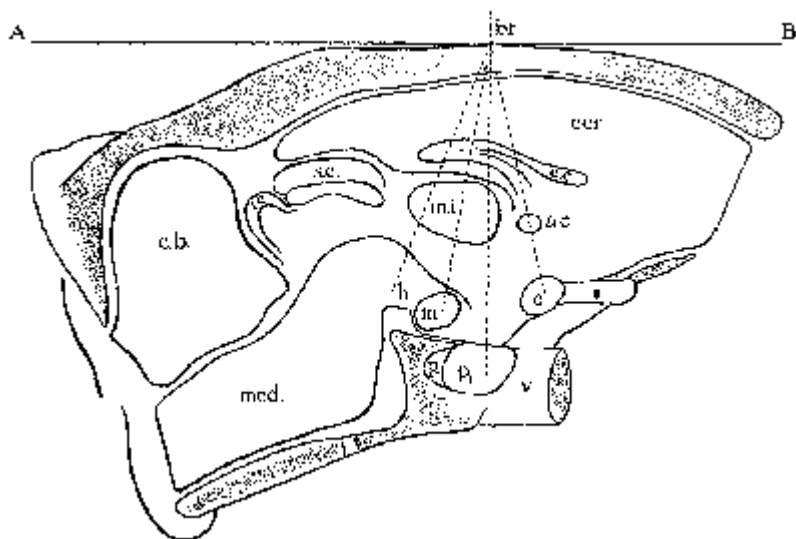
**Figure 6.1**

Diagram of a sagittal section through the cranium of a rabbit, to show the paths taken by the electrodes. ( $\times 2$  normal.) A-B, anterior-posterior tangent to the skull, through bregma; a.c., anterior commissure; br., bregma; c., optic chiasma; c.b., cerebellum; c.c., corpus callosum; cer., cerebral hemisphere; h., posterior region of hypothalamus; i.c., inferior colliculus; m., mammillary body; med., medulla; m.i., massa intermedia; p<sub>1</sub>, anterior lobe of pituitary; p<sub>2</sub>, posterior lobe of pituitary; s.c., superior colliculus; II, optic nerve; V, trigeminal nerve.

The rabbits used were fully matured does that had been separated for at least 15 days, and in most cases for 30 days.

As an anaesthetic, ether administered intratracheally was found to be most satisfactory. The trachea was easily sutured at the end of the experiment with two stitches, the only difficulty being that of cleaning the pharynx from mucous before suturing.

The method of stimulation was to insert the electrode through a small trephine hole, in the top of the skull, at bregma. It was always inserted in the mid-line, but by inclining the head at varying degrees anterior-posteriorly, different parts of the floor of the third ventricle could be stimulated.

The apparatus designed for this purpose consisted of two parts (see figures 6.10 and 6.11, plate 16); first a part for firm fixation of the head and for rotating the head into any desired position. The three points at which the head was clamped were the two posterior ends of the zygomatic arches and the upper incisor teeth. The second part was for insertion of the electrode into the brain. The electrode was carried on a vertical rack, which could slide on a pivoted arm. Thus the electrode could be moved in any plane.

To reach the various parts of the hypothalamus, the following procedure was adopted. Sagittal sections were made through the heads of a number of rabbits, which had been fixed in 10% formalin for several weeks. These were photographed, enlarged prints were made and the various measurements taken. For each point of the hypothalamus stimulated it was necessary to know two facts, first the distance of that point from

**Table 6.1**

	Distance of Points from Bregma cm.	Angle Between Tangent to Skull at Bregma and Line Joining the Point to Be Stimulated to Bregma
Optic chiasma	1.60	75°
Tuber cinereum	1.65	90°
Mammillary body	1.80	99°
Posterior hypothalamus	1.65	110°
Pituitary gland anterior lobe	2.10	90°

bregma, and secondly the angle contained between the line joining this point of the hypothalamus to bregma and a tangent of the skull drawn through bregma. (See figure 6.1.)

The average measurements obtained are given in table 6.1.

The procedure for stimulating, for example, the posterior region of the hypothalamus, was as follows:

Intratracheal cannula inserted. Rabbit's head fixed in the clamp. An incision  $2\frac{1}{2}$  in. long made in the skin at the top of the head. Head levelled transversely by means of a small oil level, placed on the two supra-orbital ridges and the two adjusting screws. The anterior-posterior tangent through the skull at bregma levelled by the same means. Head rotated through the required 20° to bring the posterior hypothalamus perpendicularly beneath bregma. A small hole, 1 mm. in

diameter, trephined through bregma, dura mater punctured. Electrode arranged with the tip directly over bregma and then racked perpendicularly down through 1.65 cm.

Various types of electrodes were used in the preliminary experiments. In the final experiments, however, unipolar stimulation was found easier to work with, since the finer electrodes could be inserted with less damage to the brain and also gave more constant results. The large diffuse electrode was a smooth brass rod inserted in the rectum, whilst the stimulating electrode consisted essentially of a fine steel wire, mounted in the holder of a hypodermic syringe needle and insulated with glass capillary tubing to within 1 mm. from the tip. The diameter of the wire was 0.26 mm. and the external diameter of the glass capillary tubing 0.37 mm. The stimulating electrode was always made the cathode. The current used for stimulating was a direct, damped, pulsating current. The final circuit used is shown in figure 6.3. Three 2.0 V accumulators were placed in series with a potentiometer, consisting of 20 m. of 20 s.w.G. eureka wire, from which a voltage varying between 3.0 and 4.0 V was tapped off. The potential difference measured across the electrodes is, of course, not the same as that tapped off the potentiometer. Voltages of 3.0, 3.5, 3.75 and 4.0 V, as tapped off the potentiometer, from A to B, are equivalent to potential differences of approximately 1.5, 1.7, 1.8 and 1.9 V across the electrodes from E to F. The currents varied between 0.4 and 0.9 mA. The contact breaker was arranged to vibrate at 5–8 c./sec. The two resistances  $R_1$  and  $R_2$  were each of 1000 ohms, and the capacity of the condenser was 4  $\mu$ F. A circuit very similar to this and its properties has been fully described by Hess (1932).

In the preliminary experiments an induction coil was used. This has one grave disadvantage. The autonomic fibres of the hypothalamus, or at least those subserving ovulation, appear to have, to undamped currents, a very high threshold of stimulation relative to the threshold of the somatic fibres in the pyramidal tracts that lie in close postero-lateral relationship to the hypothalamus. Thus it follows that to stimulate the autonomic fibres comparatively high voltages must be used, which in turn stimulate the pyramidal tracts causing the muscles of the head, neck and fore limb to twitch so that it is impossible to keep the head perfectly still. This causes the head to move relatively to the inserted electrodes and damages the brain. When damped currents are used, the threshold for the somatic fibres, though still lower than that for the autonomic fibres, is relatively much closer. Thus strong currents may be used without the disadvantageous spread of stimulation to the pyramidal tracts.

Except for the preliminary experiments, the time periods of stimulation were half a minute "on" and half a minute "off," repeated ten times, then five minutes "off." This was repeated four times, giving a total period of stimulation of 20 min. spread over 1 hr. These time periods were kept the same for all the later experiments so that a comparison could be drawn between the effects obtained from stimulation of different points.

The only symptoms noted during stimulation were a flickering of the eyelids, nictitating membranes and eyeballs. Sometimes this occurred in one eye, sometimes in both, depending on the accuracy with which the electrode was inserted in the mid-line. It is fairly certain that this effect is due to direct spread of current to the third nerve, and excitation of the somatic fibres therein. No changes in the size of the pupils were observed, so presumably the autonomic fibres in the third nerve were not stimulated. These effects were the same, whether the electrode was in the region of the tuber cinereum or passed through the hypothalamus into the pituitary gland.

At the end of stimulation, the head wound and trachea were sutured. In no case were any aseptic precautions taken, for the animals were killed before septic complications could occur. In nearly every case, uneventful and quick recoveries occurred.

The animals were killed 30–120 hr. following stimulation.

In the earlier experiments, in the cases of negative results, it was found to be difficult to say whether the animals were on heat or not. And as ovulation never occurred in an anoestrous or pregnant rabbit, the difficulty thus arose as to whether the negative effect was due to the rabbit not being on heat, or due to defective stimulation. Sections were made through the uteri of these rabbits but in several cases these were found to give insufficient evidence to decide this point. In the later experiments, the stimulated does were put with a buck immediately prior to killing and the act of copulation taken as an indication that they were on heat at the time of stimulation. The results quoted later include only those rabbits which definitely were in oestrous at killing.

The histological work consisted of sectioning the uteri in cases of negative results and the ruptured follicles in cases of positive or questionable results. 10% formalin was used as the fixative and haematoxylin with eosin as the stains.

The heads of the animals were injected with 10% formalin, and then fixed in this solution for 2–3 weeks. Careful dissection of the hardened brain then showed the point of stimulation fairly accurately, for the electrode usually left a fine trail of blood, whilst in hypothalamic stimulation around the point of stimulation

was either a yellow discoloration or a slight haemorrhage. The cause of the latter is unknown. In pituitary stimulation, the position of the electrode was gauged by the position of the haemorrhage in the gland. The extent of this varied considerably, but bore no relationship to the results of the stimulations.

### Results of Direct Stimulation of the Pituitary Gland

The pituitary gland was reached by inserting the stimulating electrode at right angles to the tangent of the skull at bregma and racking it down 2.0–2.1 cm., according to the size of the rabbit.

*In the first experiment* the stimulating electrode was a fine copper wire, s.w.g. 32, lacquered to the tip. Only the transverse section at the end was bare. This was inserted 2.0 cm. through bregma. The non-stimulating electrode was a lead knob inserted through a small incision in the soft palate to lie under the basisphenoid. Stimulation was by means of an induction coil, the secondary coil being placed as near to the primary as was possibly consistent with low spread of stimulation. The symptoms on stimulating were slight stiffening of the neck muscles, flickering of the eyelids, eyeballs and nictitating membranes and pupil constriction. The first of these is due to spread to the pyramidal tracts and the latter to spread to the third nerve. Stimulation lasted for 6 min., distributed at intervals over 1 hr. This animal was killed 51 hr. following stimulation, and on post mortem 9 ovulated follicles were discovered in the ovaries.

*In the second experiment* similar electrodes were used, but the circuit, as shown in figure 6.3, had a maximum potential difference of 3.0 V between A and B. No symptoms were observed during stimulation. Thirty hours later the animal was killed and the ovaries found to contain 6 ovulated follicles and 3 haemorrhagic follicles.

*In the third preliminary experiment* the anode was again the lead knob inserted through the soft palate, but the stimulating cathode was a steel wire insulated with glass capillary tubing (figure 6.2). The tip was bared for only  $\frac{1}{2}$  mm. instead of the usual 1 mm. Again the direct, damped form of current was used, this time with a potential difference of 4.0 V between A and B. The animal recovered quite normally but was exceptional in that it was found dead 44 hr. post-stimulation. In the ovaries were 6 ovulated follicles and 5 abnormally large follicles which it is believed were becoming cystic.

After these three experiments it was decided to use the same electrode types and stimulating currents as were being used in the concurrent experiments on hypothalamic stimulation, thus making it possible to

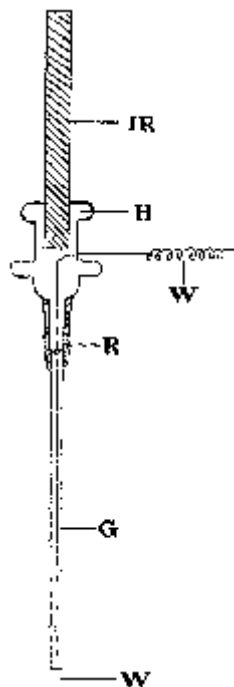


Figure 6.2

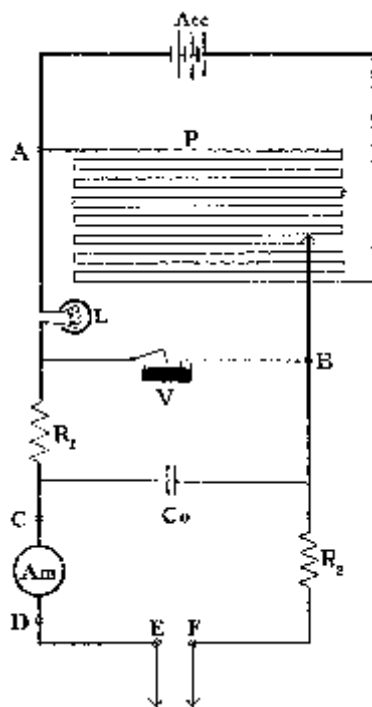
Diagram of electrode. *G*, glass capillary tubing; *H*, holder of hypodermic syringe needle; *IR*, rod of insulating material for attachment of electrode to clamp; *R*, insulating solution of rubber; *W*, steel wire.

compare the current strengths necessary to give positive results from pituitary and hypothalamic stimulation. As described previously, these electrodes consisted of a stimulating cathode of fine steel wire, insulated to within 1 mm. of the tip by glass capillary tubing, with a large diffuse anode placed in the rectum. The current was of the direct, damped, pulsating type (figure 6.3).

For the results of these experiments see table 6.2. It will be seen that stimulation with a potential difference of 3.5 V between A and B was applied to eight rabbits. Of these, seven were stimulated for the usual time of 1 hr. and showed no ovarian effects. The eighth rabbit was stimulated in exactly the same manner, but the stimulation lasted half an hour longer. Although no ovulation occurred, some secretion of pituitary hormones presumably must have taken place, for one cystic follicle and two large haemorrhagic follicles were found 75 hr. after stimulation. For the seven rabbits stimulated at 3.5 V, with the "standard" time intervals and by the "standard" method, 100% negative results were obtained.

Three rabbits stimulated with 3.75–3.8 V across A–B gave two positive results. In one case ovulation and in the other case haemorrhagic and cystic follicles were obtained.

Stimulation with 4.0 V across A–B was applied to eleven rabbits giving six positive and five negative results. That is a response of 54.5%.



**Figure 6.3**

Diagram of the final circuit used. *A-B*, voltages tapped off potentiometer, measured across these two points; *Acc*, three 2 V accumulators; *Am*, milli-ammeter; *C-D*, current passing through the animal measured across these two points; *Co*, condenser of 4  $\mu$ F capacity; *E-F*, potential difference across the electrodes, measured from these two points; *L*, small lamp of 4.5 V; *P*, potentiometer; *R*<sub>1</sub>, *R*<sub>2</sub>, resistances of 1000 ohms; *V*, contact breaker, 5–8 c./sec.

In all, effects on the ovaries of eleven rabbits have been shown after passage of electric currents of various forms through the pituitary glands. It is felt, from scanty evidence, that the most uniform effects would be obtained with an anode in the form used in the preliminary experiments inserted through the soft palate so as to lie beneath the basisphenoid bone. This condenses the current flow through the pituitary gland. In the present experiments it was necessary to have electrodes similar to those used for stimulating the hypothalamus, so that the comparison could be made between the relative strengths of current needed to elicit responses in the two regions.

It might be suggested that the presence of the needle electrode in the gland and the damage performed were sufficient to liberate enough hormone into the blood stream to cause ovulation. That this is not so is shown by the fact that stimulation with the voltage of 3.5 V gave 100% negative responses, the damage caused to the gland being as extensive in some of these experiments as in those in which positive responses were obtained by stimulation with 4.0 V.

Again, it might be suggested that the reason for negative responses is that during stimulation the insertion

of the electrode through the floor of the third ventricle damaged some structure essential to the mechanism of ovulation. To settle this point, a rabbit was stimulated in the usual manner with a voltage of 3.5 V. Fifty hours after stimulation this doe rabbit was allowed to copulate with a buck. Immediately after copulation, a laparotomy was performed and both ovaries carefully examined. There was no sign of ovulation or haemorrhagic follicles. The animal was killed 70 hr. post-stimulation, when five ovulated follicles were found in the ovaries. This demonstrates the fact that even after completion of the experiment, when the hypothalamus has been pierced, and when the pituitary gland has been damaged, the animal can still ovulate in a perfectly normal manner following copulation.

The time of ovulation in these pituitary stimulations was placed roughly between 15 and 40 hr. after stimulation. This was estimated from a study of serial sections through the ovulated follicles.

### Results of Stimulation of the Hypothalamus

In the earlier experiments, stimulation of the hypothalamus was attempted by use of the induction coil. The electrodes used were of the concentric type. The results obtained were uniformly negative for the reason given above, that is, owing to the spread of the faradic current to the somatic nerve fibres it was impossible to apply a strong enough current. They show, however, that the mere presence of the electrodes in the hypothalamus, though thicker than those eventually used, is insufficient to cause ovulation.

Later, direct pulsating damped currents were used for stimulating, obtained from the circuit already described, or similar circuits.

In the following account results obtained from the latter circuits have been classed together, and are alone considered.

#### 1. Stimulation of the Region of the Tuber Cinereum

The region of the tuber cinereum was first stimulated on the supposition that if nerve fibres which influence the anterior lobe passed via the stalk, they would converge at the tuber. The region of the tuber is taken to mean that part of the hypothalamus lying inferior to a line joining the optic chiasma to the mammillary body. This region can be reached by racking the electrode down 1.65 cm. below bregma at an angle of 90° to the tangent to the skull at this point.

In the first five experiments the concentric needle electrodes were used, the central electrode being made the cathode. The potential difference from *A* to *B* was 3.5 V. Four out of these five rabbits ovulated; in three cases haemorrhagic follicles were also formed.

Table 6.2

No. of Rabbit	Past History	Strength of Stimulating Voltage <i>A-B</i> V	Hours Between Stimulation and Killing	Ovaries on Postmortem
49	Parturition 12 days previously	3.5	73	8 moderately ripe follicles; 8 very ripe follicles
50	Parturition 18 days previously	3.5	74	10 moderately ripe follicles; 7 ripe follicles
62*	Separated 19 days	3.5 Stimulated for 1½ hr.	75	1 cystic follicle; 2 haemorrhagic follicles
63	Separated 20 days	3.5	60	2-3 old corpora lutea in each ovary; 2-3 follicles in each ovary, slightly larger than normal
71	Parturition 14 days previously	3.5	65	10 ripe follicles
72	Separated 15 days	3.5	52	7 ripe follicles
74	Parturition 8 days previously	3.5	Laparotomy at 51	Several follicles red and ripe in each ovary
75	Separated 16 days	3.5	45	9 ripe follicles
52*	Separated 21 days	3.8	74	7 ovulated follicles; 10 large follicles
60*	Separated 32 days	3.75	51	5 follicles in various stages of becoming cystic and haemorrhagic
61	Separated 34 days	3.75	51	14 ripe follicles
53	Parturition 21 days previously	4.0	73	2-3 ripe follicles in each ovary
55*	Separated 41 days	4.0	51	7 ovulated follicles; 1 follicle ovulated partly haemorrhagic
58*	Parturition 23 days previously	4.0	75	4 ovulated follicles; 1 ovulated follicle partly haemorrhagic; 4 cystic follicles; 1 haemorrhagic follicle
59*	Separated 30 days	4.0	51	10 ovulated follicles
64*	Separated 30 days	4.0	52	8 ovulated follicles; 2 large haemorrhagic follicles; 7 small haemorrhagic follicles
66*	Separated 25 days	4.0	51	2 ovulated follicles; 4 large haemorrhagic follicles; 1 very swollen follicle, partly haemorrhagic
68	Parturition 11 days previously	4.0	53	8 ripe follicles
69	Parturition 14 days previously	4.0	50	9 ripe follicles
76	Separated 22 days	4.0	42	9 ripe follicles
77	Separated 40 days	4.0	80	11 ripe follicles
78*	Separated 55 days	4.0	78	5 ovulated follicles; 3 haemorrhagic follicles

\* Indicates a positive result.

In the three preliminary experiments the results were also positive.

In the other fifteen cases, unipolar stimulation was used. The electrodes were here similar in every detail to those used in the later experiments upon the stimulation of the pituitary, that is, the stimulating cathode was as shown in figure 6.2, with a large anode in the rectum.

Two rabbits were stimulated with a potential difference of 3.0 V from *A* to *B*. One of these killed 100 hr. later showed many large cystic and haemorrhagic follicles in the ovaries (see figure 6.13, plate 17). The other gave a negative result.

Two rabbits were stimulated with a potential difference of 3.5 V from *A* to *B*. One of these killed 50 hr. later showed nine ovulated follicles and one cystic follicle turning haemorrhagic in the ovaries. The other gave a negative result.

Eleven rabbits were stimulated with a potential difference of 4.0 V from *A* to *B*. On killing, six of these

rabbits showed ovulated follicles together with various forms of cystic and haemorrhagic follicles. The other five gave negative results.

In all, twenty rabbits were stimulated around the region of the tuber cinereum, twelve giving positive results.

## 2. Stimulation of the Tuber Cinereum after Cervical Sympathectomy

In three rabbits the superior cervical ganglia and upper half of the cervical sympathetic chain were removed on both sides. Six weeks later, the tuber cinereum was stimulated, with a potential difference of 4.0 V from *A* to *B*. One of these three rabbits gave a positive response, for, on killing 72 hr. later, nine ovulated follicles and one haemorrhagic follicle were discovered in the ovaries. The other two rabbits gave negative results.



**Figure 6.4**  
Sagittal section through pituitary gland, stalk and hypothalamus. Note atrophic appearance of pituitary gland; line of lesion (indicted by two arrows); vacuoles in posterior lobe and stalk (previously filled with colloid). p, posterior lobe; i, intermediate lobe; a, anterior lobe. ( $\times 26$ .)



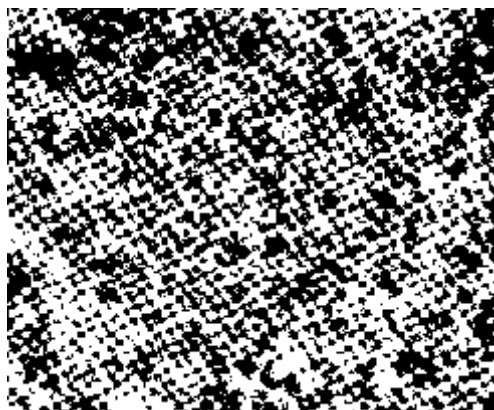
**Figure 6.5**  
Line of lesion (in figure 6.4) under higher power. Note fibrous tissue "healing", and slight accumulation of basophilic cells. B. ( $\times 72$ .)



**Figure 6.6**  
Normal posterior lobe of pituitary gland. ( $\times 360$ .)



**Figure 6.7**  
Posterior lobe of experimental animal, after stalk lesion. Note the great increase in basophilic cells and the cavities previously filled with colloid. ( $\times 360$ .)



**Figure 6.8**  
Normal anterior lobe of pituitary gland. ( $\times 360$ .)



**Figure 6.9**  
Anterior lobe of experimental animal after stalk lesion. Note reduction of the cell cytoplasm, and the apparent increase in the number of the cells. ( $\times 360$ .)

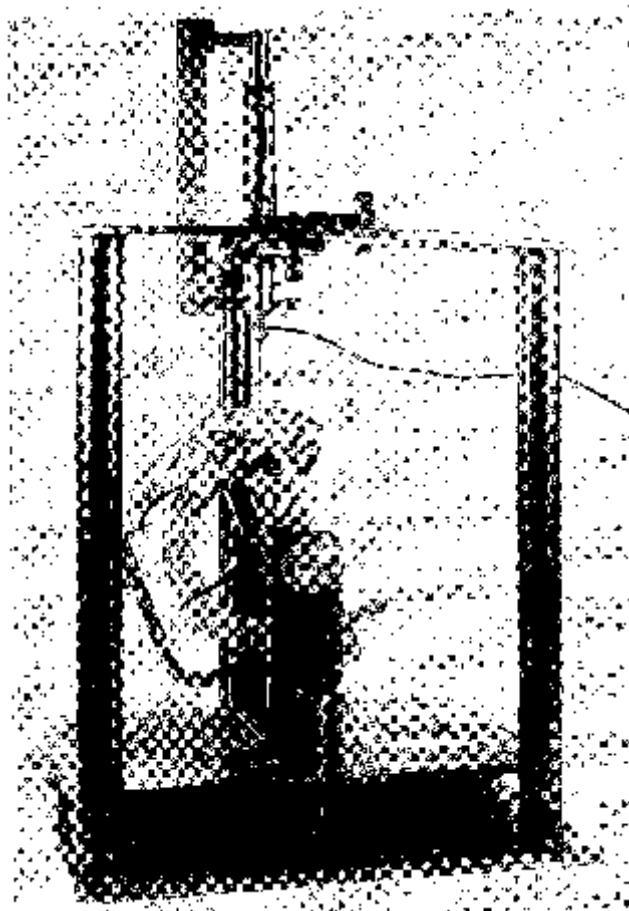


Figure 6.10

Side view of stimulating apparatus. Note that the apparatus consists of two parts—one for clamping and rotating the head, and the other for inserting the electrode. ( $\times \frac{1}{3}$ .)

### 3. Stimulation of the Posterior Hypothalamus

The exact position of the region stimulated is shown in figure 6.15, plate 17, a region not very far removed from the red nucleus and its connexions. This proximity led to complications.

Out of four rabbits stimulated in this region, three on recovery exhibited signs that the mechanism of head posture had been disturbed. In the normal standing position, the heads were rotated with the left eyes upwards. On holding the rabbits in mid-air, the left fore-limbs and hind-limbs extended, whilst the limbs of the right side flexed. It is interesting to note that the damage was mainly to the left of the mid-line in all three cases, so that the rigidity was ipsilateral. The reason for this is not clear.

These three animals showed no ovarian effects following stimulation.

The fourth animal made a very good recovery with none of the above effects. On killing 100 hr. after stimulation the ovaries were found to contain ten ovulated follicles and nine small haemorrhagic follicles. Dissec-

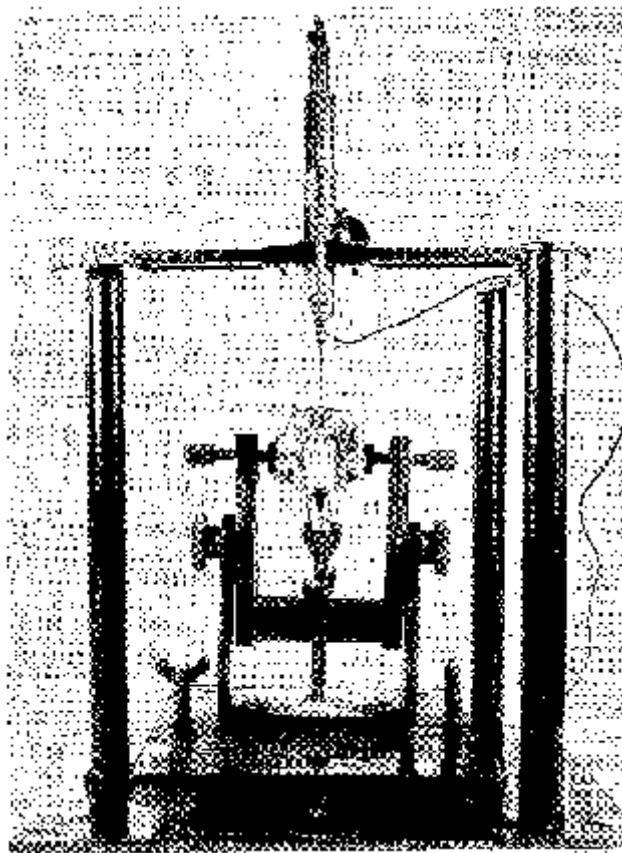


Figure 6.11

Front view of same apparatus. ( $\times \frac{1}{3}$ .)

tion of this animal's head is shown in figure 6.15, plate 17.

Thus, out of the four animals stimulated in this region, the one which recovered to normality was the one which ovulated. It is quite possible that the damage prevented the other three showing the ovarian reaction.

### 4. Stimulation of the Hypothalamus in a Ferret

Dr. F. H. A. Marshall (private communication) stimulated ferrets through the heads with the large electrical shocks that were successful in inducing ovulation in rabbits and pseudo-pregnancy in rats. With ferrets, no reaction on the ovaries could be obtained by this method. This may be due to one of two reasons. First, that ferrets normally take a very long time to copulate, any period up to 1 hr. or over. If the male is taken from the female after a quarter of an hour, ovulation may not follow. Thus to reproduce the natural conditions, it is possible that stimulation would have to be applied for an equal time. Secondly, the electrical resistance of the bones of a ferret's skull may be greater than that of rabbits and rats, in which case, not so much current would pass through the hypothalamus

and pituitary gland, for, in this method of stimulation, the electrodes were external to the cranium. With these two points in mind, it was thought to be of interest to see whether direct stimulation of the hypothalamus by the present technique would affect the ovaries, for the stimulation point would be inside the skull and the intermittent stimulation would last for 1 hr.

The electrode was inserted into the posterior region of the hypothalamus, 2 mm. above the superior surface of the pituitary gland. The potential difference between *A* and *B* was 4.0 V and stimulation was applied for the usual time intervals over 1 hr.

On recovery, this animal was discovered to have disturbed head posture similar to some of the rabbits stimulated in this region. Unlike rabbits, however, at post mortem, 102 hr. after stimulation, three cystic follicles which had undergone quite extensive lutealization were discovered in the ovaries.

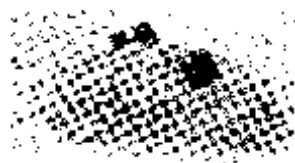
Owing to the fact that the animals were not killed until 2–3 days after stimulation, it was found difficult to place accurately the time of ovulation. From a study of sections, it appears that ovulation may occur at any time between 10 and 60 hr. following stimulation.

## Discussion

Previous workers have noted various effects on stimulation of the pituitary gland. Cyon (1898, 1899, 1900) obtained slowing of the heart with increased amplitude of the beat on stimulation by pressure or electrical excitation. Weed, Cushing and Jacobson (1913) showed stimulation of the exposed pituitary in dogs gave variation in the blood sugar. This they put down to stimulation of the posterior lobe. Keeton and Becht (1915) also found that stimulation in dogs produced glycosuria, but not if the splanchnic nerves had been previously cut. It is possible, therefore, that this effect is not due to action of the pituitary gland.

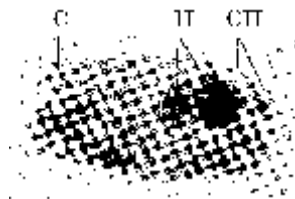
The experiments described above have shown that ovulation in the rabbit can be obtained by electrical stimulation of the pituitary and the hypothalamus. There is evidence that the effects originate definitely in the gland, or through the intermediation of the gland, for the effects upon the ovary are very similar to those obtained by the injection of extracts, either of the pituitary or of pregnancy urine. These effects, besides those of normal ovulation, include the production of cystic follicles,<sup>1</sup> of cystic follicles becoming haemorrhagic and of large haemorrhagic follicles (figures 6.12, 6.13, 6.14, plate 17).

One difference between stimulation and injection of extracts is that the injection of extracts will produce ovulation, formation of luteal tissue, and haemorrhagic follicles in the immature and anoestrous rabbit; but so far as has been observed, to obtain any result by elec-



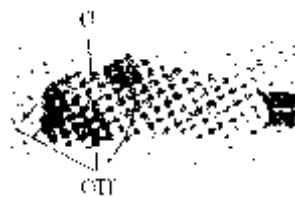
**Figure 6.12**

Ovary from rabbit 60, killed 50 hr. after stimulation of pituitary gland. Two of the follicles became very protuberant and showed some internal haemorrhage, though unruptured externally. A third follicle became haemorrhagic. ( $\times \frac{3}{2}$ )



**Figure 6.13**

Ovary from rabbit 24, killed 100 hr. after stimulation of the tuber. This ovary showed one large, clear cystic follicle, c; two cystic follicles becoming haemorrhagic, ch; and two large haemorrhagic follicles, H. ( $\times \frac{3}{2}$ )



**Figure 6.14**

Ovary from rabbit 38, killed 50 hr. after stimulation of the tuber. This ovary showed one large, clear cystic follicle, c; four cystic follicles becoming haemorrhagic, ch; and one young corpus luteum. ( $\times \frac{3}{2}$ )

trical excitation the rabbit must be well on heat. It is possible that after a more delicate technique for stimulating this gland has been obtained, prolonged stimulation over many days might produce different results.

As regards the conclusions to be drawn from the results of the hypothalamic stimulation, one difficulty arises. It is just possible that the positive results obtained were due to general spread of the stimulating current to the pituitary gland, and not due to stimulation of nerve fibres running through the hypothalamus.

At first sight the obvious experiment to perform is stimulation of this region after preliminary transection of the stalk. This is technically extremely difficult, for, as shown previously, after section of this structure, the animals enter into a state of anoestrous. Therefore, unless the stimulation was carried out immediately following the preliminary operation, the experiment would be useless. If the two operations were performed together, the time of the whole experiment and the



**Figure 6.15**

Head of rabbit 17, sagittal section. This rabbit was stimulated in the posterior hypothalamus, and killed 100 hr. later. (The ovaries showed ten young corpora lutea and nine small haemorrhagic follicles.) The dissection shows the point of stimulation quite clearly. The area surrounding the electrode showed necrosis with a pale pink blood clot. (The pink colour of the clot is greatly intensified in the photograph, appearing black in reproduction.) ( $\times 2$ .)

damage committed would be so great that almost certainly no results of value would be obtained.

Another possible way of deciding the question would be to try to define more accurately the exact regions in the hypothalamus from which the reactions could be obtained and to follow the probable nervous pathway through the mid-brain, so increasing the distance between the stimulating electrode and the pituitary gland. This would require a more delicate stereotaxic instrument than was available in the present research, and further that rabbits of the same breed and nearly uniform in size should be used, thus ensuring more accurate localization when inserting the electrode.

Concerning this question, the evidence at present available is:

1. That during hypothalamic stimulation, no changes in pupil size were observed, although the eyeballs, eyelids and nictitating membrane were seen to flicker. The conclusion drawn is that the spread of current to the oculomotor nerves was sufficient to stimulate the somatic fibres, but insufficient to stimulate the autonomic fibres which have a higher threshold of excitability. Now in the rabbit oculomotor nerves lie in closer anatomical relationship to the tuber cinereum than does the pituitary gland, so that it would be justifiable to assume that if the current spread did not excite the autonomic fibres in the third nerve it would not excite fibres of a presumably similar nature in the gland.

2. The results show that the threshold current needed to produce ovulation was the same whether the electrode was in the hypothalamus or in the pituitary. This would indicate that spread of current during hypothalamic stimulation would be insufficient to account for the results obtained.

3. In rabbit 17, stimulated in the posterior hypothalamus (see figure 6.15, plate 17), the tip of the electrode was 4–5 mm. distant from the nearest point of the pituitary gland. This animal ovulated about 35 hr. post-stimulation. After taking into consideration the fact that, in a case of intended pituitary gland stimulation with the same voltage in which the electrode was found on post-mortem to be lying outside the gland but directly adjacent to it, no ovulation was obtained, it seems safe to conclude again that the results were not due to spread of current.

Therefore, the evidence at present indicates strongly, though not certainly, that ovulation in the rabbit may be induced by stimulation of nerve fibres in the hypothalamus as well as by pituitary stimulation.

If this view be accepted, it would follow that the hypothalamus forms part of a reflex path used in stimulating the pituitary gland after sexual excitement. It therefore becomes of considerable interest to discuss the pathway in detail.

It seems highly probable that the stimulation of the pituitary directly is acting on non-medullated nerve fibres in the anterior lobe and that these fibres have a very high threshold of excitability. The functional links between the hypothalamus and the anterior lobe of the pituitary that have been suggested are, the cervical sympathetic nerves, the greater superficial petrosal nerves and the pituitary stalk. Of these, it cannot be the cervical sympathetic nerves alone, for, as previously stated, ovulation can be obtained in a normal fashion after extirpation of these nerves. It may be the greater superficial petrosal nerves, but on this point there is little evidence. It appears more probable that the functional link is the pituitary stalk. The main

evidence for this is that genital atrophy follows lesions of the tuber cinereum and pituitary stalk which has been noted by several previous workers and confirmed in the rabbit in this present account.

The hypothesis that the hypothalamus influences the anterior lobe via nervous impulses passing down the stalk is open to one obvious objection: that is, the fact that nerve fibres have never been seen passing from the posterior to the anterior lobe. By the use of the de Castro technique of staining, nerve fibres may be seen passing from the pars nervosa to the pars intermedia (Cushing 1932b). Serial sections through several rabbit pituitaries have been obtained, demonstrating this fact. It is difficult to trace these fibres to their termination owing to their extremely fine character. It may be mentioned in passing that although the pars nervosa and intermedia may take the stain perfectly, the pars anterior takes it poorly. In order to surmount the above objection, it is necessary to suppose either that the nerve fibres seen to enter the pars intermedia eventually pass round the cleft into the anterior lobe, or else that the posterior or intermediate lobe can influence the anterior lobe hormonally. The former supposition is felt to be the more probable of the two.

This suggested pathway (hypothalamus, stalk, posterior lobe, anterior lobe) may possibly be active in several phenomena which have previously been difficult to explain. It is well known that in some birds and in the ferret, the sex cycle may be influenced by extra radiation. It is probable that the radiation, at least in the ferret, acts on the anterior lobe of the pituitary through the intermediation of the eyes (Bissonnette 1936). Collin (1935) has brought forward evidence that there is a nervous connexion passing from the optic tract through the hypothalamus to the stalk of the pituitary. On correlation, these two facts fit in well with the above theory.

Again, it is highly probable that the uterus may affect the anterior lobe of the pituitary gland by nervous reflex paths, for it has been shown that hysterectomy in the pseudo-pregnant rabbit, or guinea-pig, will prolong the life of corpora lutea in the ovaries (Loeb 1923, 1927; Loeb and Smith 1936; Asdell and Hammond 1933). Selye (1934) showed in rats that Caesarian section initiates lactation and the recurrence of oestrous cycles, though this does not occur if the uterus is distended with wax after removal of the foeti.

From the work of Selye and McKeown (1934), it appears that mechanical stimulation of the nipples is the cause of lactation dioestrus in rats and mice.

Thus there is evidence that the anterior lobe may be influenced by nervous effects from the eyes, uterus and mammary glands, as well as from the vaginal region. Also, Haterius (1933) produced evidence that a

psychic factor normally plays a part in the induction of pseudo-pregnancy in rats, whilst Theobald (1936), who has collected clinical evidence for a diencephalic centre governing the menstrual cycle in women, states that psychic factors such as fear of pregnancy and hypnosis may affect this cycle through the supposed centre.

It might be theorized that the hypothalamus controls the secretion of hormones, other than the gonadotropic hormone, from the anterior lobe. In a recent review on the relationship between the hypothalamus and the pituitary, Dodds and Noble (1936) draw attention to the well-known facts that hypothalamic damage may cause glycosuria, adiposity, and genital atrophy, which are possibly all anterior lobe effects. Here then is evidence that the hypothalamus controls the secretion of the hormones influencing sugar and fat metabolism. There is no reason to doubt that the thyrotropic, adrenotropic, laetogenic, parathyrotropic and growth hormones are not similarly controlled.

Finally, much evidence has accumulated in the past few years indicating that the hypothalamus contains centres controlling the autonomic nervous system. If it is further shown that the pituitary gland and, through the intermediation of the structure, the thyroid, parathyroids, adrenals and gonads are likewise influenced by this important region of the brain, then it would be possible to say that a very large part of both the nervous and chemical links which unite one part of the body functionally with another part are controlled by this region of the diencephalon.

I wish to express my sincerest thanks to Professor H. A. Harris and Professor E. D. Adrian for their ever willing advice. In particular, my deepest gratitude is due to Dr. F. H. A. Marshall, first, for suggesting this work to me, and secondly for his constant aid and encouragement. Also I should like to thank Mr. E. Powell for the care he bestowed on the animals.

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### Summary

Lesions in the stalk of the pituitary gland have been shown to cause genital atrophy in male and female rabbits.

Direct stimulation of the pituitary gland in female rabbits induced ovulation 15–40 hr. later and sometimes the formation of cystic and haemorrhagic follicles.

Stimulation of the hypothalamus gave results similar to the above. Evidence is adduced that this is an effect on nerve fibres in the hypothalamus rather than due to spread to the pituitary gland.

The control of the anterior lobe of the pituitary by the hypothalamus is discussed, with particular reference to the nervous pathway involved.

### Note

1. One observation may be of agricultural interest. In one animal (rabbit 60), cystic and haemorrhagic follicles were obtained following pituitary stimulation (figure 6.12, plate 17). Fifty hours after stimulation and just before being killed, this rabbit was put with a buck. The experimental animal showed intense sexual excitement, manifested by continuous "jumping" on to the male rabbit. The condition produced by this stimulation is thus very suggestive of the pathological condition, nymphomania.

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The pathway by which the hypothalamus influences the secretion of the anterior pituitary gland has been the subject of much research. The possibility that the hypophysial portal vessels may form part of this pathway has been discussed,<sup>1</sup> and the anatomy of these structures in the rat and other forms has been described. In 1949, Harris<sup>2</sup> observed that regeneration of the hypophysial portal vessels in the rat may follow section of the pituitary stalk, and later<sup>3</sup> that post-operative return of reproductive activity can be correlated with such regeneration. In this work the hypophysial stalk was exposed by the sub-temporal route and either cut with a knife or torn with a hook. In the literature, accounts of stalk section made by a variety of methods show divergent results. Since the conditions for a regeneration of the portal vessels may vary according to the method used for severing the hypothalamo-hypophysial connexions, we have investigated the regenerative capacity of these vessels in different circumstances.

Regeneration of the hypophysial portal vessels and the state of the endocrine glands have been studied in rats. The experiments are divided into four groups:

1. Hypophysectomy and transplantation of anterior lobe tissue under the median eminence (12 experiments).
2. Hypophysectomy and transplantation of the extirpated anterior lobe into the emptied hypophysial capsule (16 experiments).
3. Extirpation of the anterior pole of the hypophysis (13 experiments).
4. Section of the hypophysial stalk (25 experiments).

Twelve hypophysectomized rats served as control material. The duration of the experiments varied from 1 to 104 days.

The observations show that a hypophysial transplant near the hypophysial stalk *in the subarachnoid space* becomes richly vascularized from the portal vessels. Regeneration of the portal vessels after stalk section by the sub-temporal route has been confirmed, and previous studies of the activity of the anterior lobe<sup>3</sup> extended. It seems clear that in the subarachnoid space

the portal vessels can undergo proliferative and reparative changes. Further work is in progress concerning the cytology and activity of these transplants.

The evidence obtained from the experiments on rats with a transplant in the hypophysial capsule, and on rats with the anterior pole of the hypophysis removed, indicated that capillaries derived from the portal vessels may invade such isolated anterior lobe tissue *in the capsule*. However, anterior pituitary tissue transplanted into the capsule did not become vascularized or active. Since the possibilities of obtaining living transplants of anterior lobe tissue inside the hypophysial capsule are highly dependent on technical factors, other techniques are being investigated.

#### Notes

1. Harris, G. W., *Physiol. Rev.*, 28, 139 (1948).
2. Harris, G. W., *Nature*, 163, 70 (1949).
3. Harris, G. W., *J. Endocrinol.*, 6, xviii (1949a).



It has been known since the time of Heape (1905) that the isolated female rabbit does not ovulate spontaneously but requires sensory stimuli, normally associated with coitus, to excite follicular rupture. The evidence indicates that the act of mating excites a nervous reflex pathway passing to the hypothalamus, which in turn stimulates the anterior pituitary gland to release increased amounts of luteinizing hormone (LH). Harris (1937, 1948), Haterius & Derbyshire (1937) and Markee, Sawvyer & Hollinshead (1946) all found that electrical stimulation of the hypothalamus in the isolated female rabbit was followed by ovulation.

The mechanism by which the hypothalamus regulates gonadotrophin secretion by the anterior pituitary gland seems to involve the liberation of a humoral agent from the nervous tissue of the median eminence of the tuber cinereum into the hypophyseal portal vessels, and thereby the transmission of this agent to the anterior pituitary gland. Evidence relating to this view is as follows (see Harris, 1955, for detailed discussion): (1) It is very doubtful whether nerve fibres, other than vasomotor, exist in the adeno-hypophysis. (2) All major vertebrate groups possess a vascular system carrying blood *from* the median eminence *to* the adeno-hypophysis (Green, 1951). (3) The evidence that electrical stimulation of the hypothalamus evokes ovulation in the rabbit, whereas similar stimulation applied directly to the anterior pituitary gland itself is ineffective (Markee et al. 1946; Harris, 1948), is compatible with the view of humoral stimulation of the gland cells. (4) Permanent interruption of the hypophyseal portal vessels (by pituitary stalk section or pituitary transplantation) results in a permanent and severe loss of ovarian activity; temporary interruption followed by regeneration of the portal vessels is often associated with a post-operative return of normal ovarian function.

The present investigation was planned to see whether any material with excitatory actions on anterior pituitary cells could be extracted from the median eminence of the tuber cinereum. The median eminence is phylogenetically the most ancient part of the neurohypophysis. It is also the zone where hypothalamic nerve fibres enter into intimate association with the primary

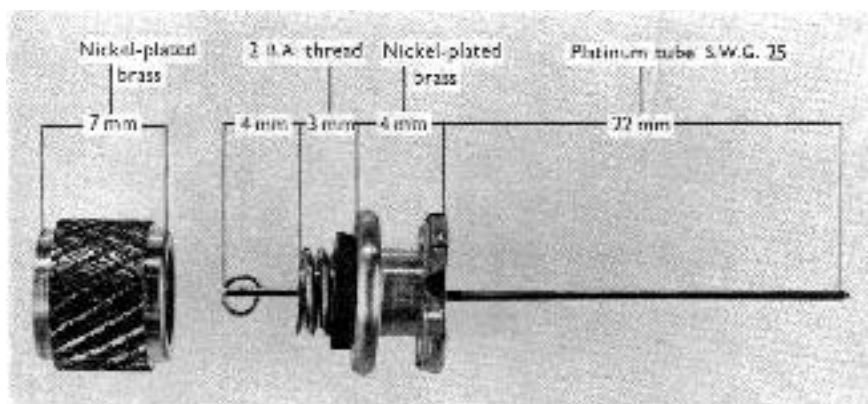
capillaries of the hypophyseal portal vessels. Thus if the hypothalamic nerve tracts associated with ovulation in the rabbit contain some humoral agent concerned with gonadotrophic secretion, it would be reasonable to suppose that a more concentrated extract (per unit weight of tissue) could be obtained from the median eminence than from the hypothalamus. Extracts of median eminence have been tested for gonadotrophin-releasing properties by means of intrapituitary infusions, with a technique similar to that of von Euler & Holmgren (1956). Ovulation, as shown by the presence of ruptured follicles in histological sections of the ovaries removed 48 hr after infusion, was taken to indicate stimulation of secretion of gonadotrophic hormone.

### Methods

Adult female rabbits, 2.0–4.0 kg body weight, were used. Two hundred and fourteen were Chinchillas and eleven were of other breeds. Diet consisted of pellets (M.R.C. diet 18, supplied by A. C. Taylor Ltd) and occasional vegetables and bread, with tap water ad lib.

**Pituitary Cannula (Pl. 1, figure 8.1)** The cannula consisted of a length of platinum tube (SWG 25) about 35 mm long. This was mounted in a cylindrical brass collar bearing two circular flanges. The upper part of the collar was screw-threaded for attachment of a protective screw cap (with rubber washer). A platinum stilette that protruded slightly from the lower end of the cannula was kept in situ except during infusions.

**Implanation of the Cannulae** The cannulae were implanted with the aid of the stereotaxic instrument described and illustrated by Harris & Woods (1958; plate 2). Anaesthesia was induced with pentobarbitone sodium (Nembutal, Abbott Laboratories), 35 mg/kg body wt, i.v., and the rabbit's head orientated in the stereotaxic instrument. A mid-line incision was made in the shaved scalp, a small trephine hole bored with a dental drill at the bregma and three screws (3/16 in. (4.5 mm), 6 B.A. thread, "Staybrite" 18/3 FMB steel)



**Figure 8.1**

Photograph of a cannula. A 35 mm length of platinum tube (s.w.g. 25) is carried by a brass collar soldered near one end of the tube. The collar bears two flanges and a screw thread on its upper end. A protective screw cap and rubber washer are also shown. Except during infusion, the platinum stilette keeps the cannula patent.

inserted into the skull a few millimetres from the trephine hole. The cannula was then lowered into the region of the tuber cinereum according to the coordinates given by Harris (1937), a lateral X-ray photograph of the head was taken, and the alignment of the cannula with the sella turcica measured. If antero-posterior adjustment of the cannula position was necessary, it was removed from the brain and reinserted. After correct alignment the cannula was lowered a further distance (as measured on the X-ray) so that its tip was situated in the anterior two-thirds of the sella turcica. A final X-ray photograph was taken for reference (see Pl. 1, figure 8.2). This method of inserting the cannula gave good accuracy in placing the cannula tip in the pars distalis with only a single puncture of the gland. Once the cannula was in the desired position, dental cement was applied as a mound to incorporate the lower flange of the cannula collar and the three fixing screws in the skull. After applying antibiotics (procaine and sodium penicillin and streptomycin sulphate ("Seclomycin," Glaxo Laboratories)) to the wound the scalp was sutured around the collar of the cannula with interrupted eversion sutures, the protruding cannula tube covered with a protective cap, and the animal injected subcutaneously with "Seclomycin." The wounds healed without difficulty, sepsis did not occur, the cannulae did not move, and the rabbits showed no sign of inconvenience from the implanted units.

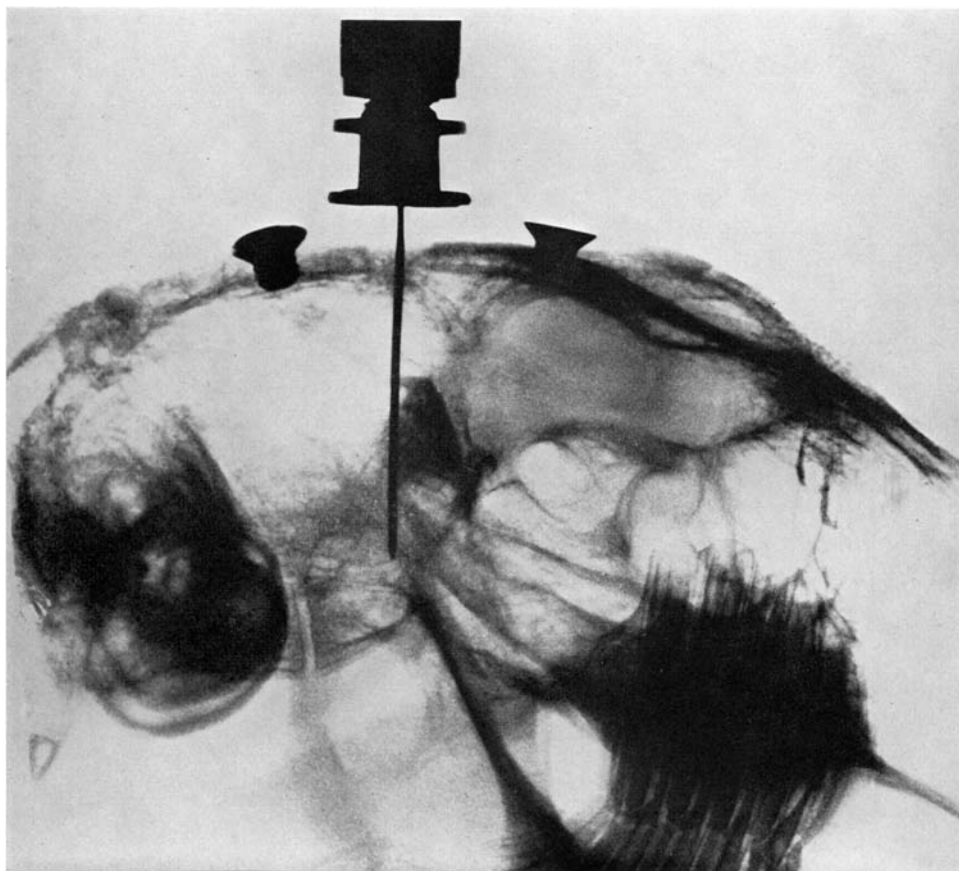
**Intrapituitary Infusions** Infusions of various substances into the pituitary gland were carried out not less than 1 week after implantation of the cannulae. The longest interval between implantation and infusion was 21 days. The animals were brought into the laboratory and placed, conscious and unrestrained, in a small cage on the bench. The fluid for infusion was drawn into a length of polythene tubing (Portex tubing, No.

50, 0.5 mm bore) so as to leave about 1.5 cm air space at one end. This end was then pushed on to a needle attached to an all-glass hypodermic syringe (0.25 ml. Tuberculin, Summit) filled with liquid paraffin. The syringe was clamped in a Palmer Slow Injection apparatus and a small amount of paraffin injected into the polythene tubing in order to observe the free flow of the fluid to be infused and to measure the length of the air bubble. The other end of the polythene tubing, which had previously been slightly tapered in a flame, was then attached to the protruding cannula tube after removal of the protective cap and stilette. After starting the infusion the air bubble moved slowly along the tubing, a useful indication that the very slow infusion (0.134 ml./2 hr) was proceeding as desired. The length of the air bubble served to indicate the pressure relations of the system. All infusions of different substances were given in 0.134 ml. over a 2 hr period (see below).

**Intravenous Infusions** For experiments involving systemic administration the various materials were expressed from an all-glass hypodermic syringe (5 ml. B-D Yale, Luer-Lok) by a Palmer Slow Injection machine into a length of polythene tubing similar to that used for the intrapituitary infusions. The free end of the polythene tubing was attached to a short length of hypodermic needle which was inserted into the marginal vein of the ear and held in place by a strip of adhesive tape. The volume of fluid infused intravenously over a 2 hr period was 2.1 ml. The movement of the syringe plunger was sufficient indication that the infusion was proceeding as desired.

#### Preparation of Extracts of Brain Tissue

**Rabbit Brain** Oestrous female Chinchilla rabbits, that had been isolated in the laboratory for more than a



**Figure 8.2**

X-ray photograph, lateral view, of a rabbit's head with an implanted cannula. Note the stainless-steel screws in the vault of the skull. The cannula is fixed to these screws with a mound of dental cement (not visible in a radiograph). The tip of the cannula is situated in the sella turcica. The apparently enlarged diameter of the intracranial part of the cannula is due to X-ray scatter.

month, were anaesthetized with ether and killed by sawing the head across transversely in a plane just caudal to the sella turcica. The median eminence and other brain tissues were removed and immediately frozen with CO<sub>2</sub> snow. Each extract of rabbit tissue was prepared by pooling samples from 4 animals.

**Monkey Brain** The heads of 25 Macaque monkeys, of mixed sexes and age groups, were dissected about 1 hr after the animals had been killed with nitrous oxide. The median eminences were pooled and frozen in CO<sub>2</sub> snow.

**Cattle Brains** The heads of steers, killed by cutting the throat, were dissected in a slaughter house. Approximately 1–2 hr elapsed between death and dissection. The vault of the cranium was removed with an axe, the dura opened and brain samples collected in the following order: cerebral cortex, corpus callosum, caudate nucleus and median eminence of the tuber cinereum. Great care was exercised to avoid taking any anterior pituitary tissue with the median eminence. With the

use of long curved scissors the stalk was cut 1 or 2 cm above the level of the diaphragma sellae. The average weight of a single cattle median eminence was 100.3 mg (range of average weights in the ten different pooled batches was 90.5–125.6 mg). After dissection each brain sample was immediately frozen with CO<sub>2</sub> snow. Each of the ten cattle extracts, referred to below, was made by pooling samples from 10–24 brains.

**Extraction Procedures** The frozen tissues were thawed and then homogenized in 0.5% acetic acid or 0.5% hydrochloric acid in a Potter homogenizer at 0° C for 1–2 min. With some batches the homogenate was divided into two equal parts, and one part placed in a boiling water-bath for 5–10 min. The boiled and unboiled homogenates were then centrifuged for 10 min at 3000–4000 rev/min at 0° C. The supernatants were neutralized and made isotonic by addition of solid NaHCO<sub>3</sub> and NaCl (in the case of the acetic acid extracts) or by addition of solid NaHCO<sub>3</sub> alone (in the case of the hydrochloric acid extracts). Any precipitate formed was removed by centrifugation. The final

volume of these extracts was adjusted so that 1 ml. of extract was equivalent to 160 mg of brain tissue (wet weight). The extracts were distributed and sealed in 1 ml. glass vials and stored in a deep-freeze. The solutions were clear or pale yellow, though sometimes a slight flocculent precipitate formed.

### Drugs

The following drugs were used: Synthetic lysine vasopressin (L 8-vasopressin, Sandoz A. G., Basel, Batch Nos. L 8/006/02 and L 8/005/02); synthetic oxytocin (OTS 68, Sandoz A. G., Basel, Batch No. 69005); adrenaline bitartrate (British Drug Houses); histamine dihydrochloride (British Drug Houses); serotonin creatinine sulphate (L. Light and Co. Ltd.); tartaric acid (British Drug Houses). Synthetic lysine vasotocin was presented to us by Sandoz A. G., Basel, and substance P by Professor J. H. Gaddum.

### General Plan of Experiments

After the rabbits had been isolated for 3–4 weeks the pituitary cannulae were implanted. Seven or more days later the intrapituitary infusions were carried out under quiet conditions and with the minimum of handling to avoid “spontaneous” ovulation. Intravenous infusions were also made at the end of a similar isolation period. Forty-eight hours after infusion the animals were killed with an overdose of Nembutal and the ovaries and uterus examined macroscopically before being placed in formol-saline for subsequent histological study. Only the presence of ruptured follicles was taken as a positive result, haemorrhagic follicles alone in the ovaries being recorded as a negative response.

**Histology** Following fixation in 10% formol-saline, dehydration and embedding in paraffin wax, the ovaries were serially sectioned. After removing the skin, lower jaw and orbital contents, the base of the skull underlying the pituitary capsule was removed, and the heads (with cannulae in situ) were placed in 10% formol-saline. When fixation was complete the dental cement on the cranial vault was dissolved in chloroform, the cannulae removed and the skulls decalcified in equal parts of 40% formic acid and 7% sodium formate solution. Blocks of tissue containing the hypothalamus, pituitary gland and base of skull were serially sectioned at 5–10  $\mu$  in the horizontal plane. All tissues were stained with haematoxylin and eosin.

### Results

#### Preliminary Experiments

In order to establish suitable conditions for infusions into the pituitary gland, preliminary experiments were carried out as follows:

a. Cannulae of different external diameters were implanted in the pituitary and about 1 week later the animals were killed and their pituitaries sectioned for histological study. Little sign of damage to the gland tissue surrounding the cannula tip was observed when platinum tubing of an external diameter of 0.5 mm (S.W.G. 25) was used to construct the cannulae.

b. An infusion period of 2 hr was used throughout all experiments for two reasons: first, the physiological process by which the release of luteinizing hormone is excited from the anterior pituitary of the rabbit following the stimulus of coitus may be a slow process of about  $\frac{1}{2}$ –1 hr (Fee & Parkes, 1929; Westman & Jacobsohn, 1940); and secondly, for any given rate of infusion longer periods allow a greater over-all volume to be administered. This greater volume facilitates both the extraction of small amounts of tissue and administration of the extracts. With the standard 2 hr period solutions were infused at different rates and the pituitary glands examined histologically. It was found that rates of 2.24  $\mu$ l./min, or greater, were accompanied by signs of damage to the surrounding gland tissue, whereas damage was absent or very slight at an infusion rate of 1.12  $\mu$ l./min. The latter rate was adopted for all experiments. The total volume infused over the 2 hr periods was therefore 134  $\mu$ l., which may be compared with the dead space of a cannula of 1.1  $\mu$ l. When infusing such small volumes at such slow rates care must be taken to ensure that no leak occurs at the junction of the polythene tubing with the platinum cannula, and that the cannula is not wholly or partially blocked with fibrous tissue. Leakage at the junction of the tubing is easily seen. Any blockage of the cannula, with a resultant increase in pressure in the infusion system, can easily be ascertained by observing the length of the air bubble in the polythene tubing during an infusion period.

c. The volume of anterior pituitary tissue permeated by infused fluid was studied by infusing solutions of toluidine or aniline blue dyes. At the end of the infusion period the animals were killed and frozen sections made through the pituitary glands. Visibly stained tissue was found to occupy an oval area around the tip of the cannula and approximate measurements indicated that up to 40% of anterior pituitary tissue was stained. The speed with which infused solutions pass from the gland into the general circulation was studied by infusing solutions of radioactive iodine into the pituitary for periods of  $\frac{1}{2}$ –1 hr, killing the animal immediately infusion was stopped and quickly removing the pituitary gland. The radioactivity in the whole pituitary gland (measured in a well-type scintillation counter) was found to be equivalent to the radioactiv-

ity contained in an amount of infusion fluid passing into the gland in 30 sec. It may be commented that solutions of proteins, fats or other substances may diffuse into, or be absorbed from, pituitary tissue at different rates from dyes or radioactive iodine.

From these preliminary experiments it was decided to use, in all the experiments described below, platinum cannulae with an external diameter of 0.5 mm, and to infuse all solutions into the pituitary gland over a 2 hr period at a rate of 1.12  $\mu$ l./min. All intravenous infusions were given over a 2 hr period, a total of 2.1 ml. being administered.

In the results presented below only those animals are included in which it was verified histologically that the cannula tip had been in some part of the pars distalis, and in which signs of damage to surrounding anterior pituitary were absent. Positive results include only those animals in which freshly ruptured follicles were found to be present in the ovaries on histological examination.

#### Infusion of Rabbit Median-Eminence Extract

Infusion of extracts of median-eminence tissue obtained from adult oestrous female rabbits was made into the pituitary gland of 4 recipient rabbits. Since the median eminence of the rabbit is too small to remove without taking some adjacent hypothalamic tissue, it is impossible to express the dose of extract in terms of equivalent wet weight of fresh median-eminence tissue. The dose is therefore expressed as the number of median eminences contained in the volume of extract infused. Out of the 4 rabbits infused with a dose of extract equivalent to 1.4–1.8 median eminences into the pituitary gland, 3 ovulated. Two rabbits were each infused with a dose of extract equivalent to 2.0 median eminences intravenously; neither ovulated.

#### Infusion of Monkey Median-Eminence Extract

Eight rabbits received an intrapituitary infusion of an extract of median-eminence tissue obtained from monkeys. The dose infused was equivalent to 20 mg wet weight of fresh tissue. Four out of the 8 rabbits ovulated.

#### Infusion of Cattle Median-Eminence Extract

Extracts of median-eminence tissue obtained from cattle were infused, at various dose levels, into the pituitary glands of 84 rabbits. In 14 cases the extract had not been subjected to boiling during the extraction procedure, but since the results did not differ from those obtained with boiled extracts the results of the experiments with both types of extracts are grouped together and presented in table 8.1. This table also shows the results obtained following *intravenous* infusions of the same extracts into 32 rabbits.

**Table 8.1**

The effect of intrapituitary and intravenous infusion of cattle median-eminence extract on ovulation in the rabbit

Dose of Extract (mg Wet Wt. of Tissue)	Intrapituitary		Intravenous	
	No. of Animals	No. Ovulating	No. of Animals	No. Ovulating
100	—	—	7	1
50	—	—	16	5
20	28	18	9	0
10	14	10	—	—
5	14	9	—	—
2.5	22	11	—	—
1.0	6	0	—	—

**Table 8.2**

The effect of intrapituitary infusions of control extracts and solutions on ovulation in the rabbit

Substance	Dose	No. of Animals	No. Ovulating
Cerebral cortex	20	(mg wet wt. of tissue)	0
Corpus callosum	20		0
Caudate nucleus	20		1
Hypothalamus (not including median eminence)	6		0
Synthetic lysine-vasopressin	0.134–2.68 u.	9	0
Synthetic oxytocin	0.4 u.	8	0
Synthetic vasotocin	0.48–2.68 (pressor) u.	9	0
Adrenaline bitartrate	120 $\mu$ g*	11	2
Histamine dihydrochloride	120 $\mu$ g*	9	2
Serotonin	1 mg*	7	1
Substance P	20 u.	8	0
		95	6

\* Dose expressed as wt. of salt.

#### Infusion of Control Brain Extracts

Extracts were prepared from the parieto-occipital cerebral cortex, corpus callosum, caudate nucleus and hypothalamus (not including the median eminence) of rabbit and cattle brain, according to the procedure used for extracting median eminence tissue. Intrapituitary infusion of these extracts was carried out in a manner identical to that used for median-eminence extracts. The results of these and other control experiments are presented in table 8.2.

#### Infusion of Other Control Substances

In view of the known presence of various substances in the median-eminence region of the brain, intrapituitary infusion of some of these substances was made under the usual standard conditions. The results of these

experiments are given in table 8.2. It may be pointed out that some of the ovulation responses following infusions of adrenaline, histamine and serotonin were probably dependent upon the acidity of the solution used. Intrapituitary infusion of adrenaline bitartrate solution (pH 3.5) was followed by ovulation in 1 out of 3 animals. Similar infusions of neutralized solution resulted in ovulation in only 1 out of 8 rabbits. Intrapituitary infusion of histamine dihydrochloride solution (pH 4.3) into 1 rabbit was followed by ovulation. Only 1 out of 8 animals ovulated following similar infusions of neutralized solution. Intrapituitary infusion of serotonin creatinine sulphate solution (pH 3.5) was followed by ovulation in 1 out of 3 animals. Similar infusions of neutralized solution did not evoke ovulation in any of 4 rabbits.

Thus out of 90 rabbits receiving an effective dose of rabbit, monkey or cattle median-eminence extract into the pituitary, 55 ovulated. Out of 95 rabbits receiving an intrapituitary infusion of control brain extracts and other control substances, only 6 ovulated. Comparing these figures,  $\chi^2 = 60.3$  and  $P < 0.001$ . (Out of 67 isolated female rabbits maintained in the same animal colony and killed for other purposes, 6 were found to have corpora lutea present in the ovaries.)

## Discussion

A technique for administering minute volumes of various substances directly into the pituitary gland of conscious rabbits was described by von Euler & Holmgren (1956). In the present study various modifications of this method were found useful. In the first place, prolonged infusions over 2 hr periods given at a rate of  $1.12 \mu\text{l./min}$  were employed here, instead of repeated injections of about  $1.0 \mu\text{l}$ . This necessitated a different coupling between the implanted cannula and the syringe or injection apparatus. It was found convenient to extend the platinum tube forming the cannula through the collar, so that direct attachment of polythene tubing could be made to the cannula. A screw cap was used to protect this short exteriorized length of the cannula. Secondly, since it was decided to use each rabbit for only one infusion, the guide system of von Euler & Holmgren (1956), which allows removal and replacement of a cannula, was unnecessary and in the present work the cannula itself was implanted and fixed chronically in place. In a few preliminary experiments the tip of the cannula was found to become blocked by fibrous tissue in the week that elapsed between implantation of the cannula and the infusion. For this reason a fine platinum-wire stilette, of such a length that it just protruded from the lower end, was left in the cannula until just before an infusion. As a

check on blockage of the infusion system, an air bubble placed between the oil and infused solutions in the polythene tubing served to indicate (a) any back pressure (length of the air bubble), and (b) the progress of the infusion (length of tubing traversed by the bubble). Some such indicator is necessary because it is possible, with a blocked cannula, for the 60 cm of polythene tubing to distend sufficiently to accommodate the small volume of fluid thought to be entering the pituitary gland. Thirdly, the use of X-ray control during the insertion of the cannulae allowed re-alignment if the stereotaxic positioning was in error and gave good accuracy in placing the cannula tip in the pars distalis of the adenohypophysis. Histological check of the cannula position showed that the tip was in the pars distalis in 227 rabbits out of a total of 247 operated upon for this and other studies.

Possible damage to anterior pituitary tissue, with consequent leakage of stored hormone from the damaged tissue into the general circulation, is an important factor to consider in analysing the results of any experiments involving injection or infusion into the gland. The compact nature of the gland tissue, and the fact that it is surrounded by a tough fibrous capsule and (in the rabbit) a bony sella turcica, are factors which render the gland liable to ischaemic necrosis if solutions are injected or infused at too high a rate. Other factors which might occasionally result in damage to the gland are injury of pituitary vessels at the time of insertion of the cannula, or infusions of solutions which are not carefully neutralized or made isotonic. Out of 212 infusions into the pituitary gland in the present study, 21 animals were discarded since the gland showed histological signs of damage. [13 of these had received infusions of median eminence extract (7 ovulated), whilst 8 had received infusions of control substances (1 ovulated).]

Out of 90 rabbits infused with an effective dose ( $>1.0 \text{ mg}$ ) of extract of median-eminence tissue into the pituitary, 55 ovulated; in comparison similar infusions of control extracts in 95 rabbits resulted in ovulation in only 6. In considering the significance of these findings, attention should be paid to the following points:

**Occurrence of "Spontaneous" Ovulation** As mentioned above, out of 67 isolated female rabbits, maintained under the same conditions as the present experimental animals and killed for other purposes, 6 were found to have corpora lutea of various ages in the ovaries. From the size and appearance of most of these corpora it was possible to distinguish them from freshly ruptured follicles. Thus it is likely that the occurrence of spontaneous ovulation is a cause of error in only 2–3% of

experiments; a figure approximating to the proportion of ovulation responses seen in the control animals.

**Back-Tracking of Solutions Infused into the Pituitary along the Cannula to Stimulate Nerve Fibres in the Median Eminence** This might indirectly excite release of LH. It seems unlikely that the present results can be attributed to such back-tracking of solutions, since the control intrapituitary infusions were not followed by a significant number of ovulatory responses.

**Vasomotor Effects Exerted by Infused Solutions on Pituitary Blood Vessels** Worthington (1960) has described the effects of various drugs and physical traumata on the hypophyseal portal circulation. It is likely that extracts of median-eminence tissue contain certain substances (such as vasopressin and histamine) capable of eliciting vasomotor effects, and it is possible that disturbances of the pituitary circulation by such vasoactive substances were responsible for eliciting increased secretion of luteinizing hormone. Such a possibility is rendered unlikely by the negative results seen following infusions of vasopressin, histamine and adrenaline (only 4 out of 29 rabbits ovulated following infusion with large doses of these substances).

**Damage to Anterior Pituitary Cells with Consequent Leakage of Stored LH into the General Circulation** Although the results of any experiments in which the pituitary was found to show histological signs of damage have been rejected, the possibility remains that the ovulation responses were evoked by undetected cellular damage. This is unlikely, since intrapituitary infusions of control brain extracts (prepared, neutralized, made isotonic and infused in an identical way to the median-eminence extracts) did not excite similar responses, and since intravenous infusions of 50–100 mg of median eminence resulted in ovulation in 6 out of 23 rabbits.

**Contamination of the Median-Eminence Extracts with LH** Although some contamination of the median-eminence extracts with LH is possible, it is unlikely that the results obtained on intrapituitary infusion of median-eminence extracts are due to such contamination. A dose of 2.5 mg median-eminence extract infused into the pituitary excited ovulation in 11 out of 22 rabbits, but the intravenous infusion of 20 mg median-eminence extract did not cause ovulation in any of 9 animals. A further fact to consider in this connexion is that the activity of 2.5 mg of median-eminence extract, given by intrapituitary infusion, is not lost following exposure to 100° C for 10 min.

It is probable therefore that there is some substance in extracts of the median eminence which is active in

exciting the secretion of luteinizing hormone from anterior pituitary cells in the rabbit. A similar substance, or activity, has not been detected in extracts of cerebral cortex, corpus callosum or caudate nucleus.

Whilst the work described above was in progress a similar study on rats was being undertaken in the same department by Dr. Nikitovitch-Winer (Nikitovitch-Winer, 1962). This worker infused the same cattle extracts as those used in the present study on rabbits, and also extracts of the median eminence of rats, into the pituitary gland of pro-oestrous female rats in which spontaneous ovulation had been blocked by administration of pentobarbitone sodium. It was found that infusion of cattle and rat median eminence extract into the pituitary resulted in ovulation in 26 out of 34 animals studied. The effective dose was approximately 1 mg (wet weight) of median eminence tissue, irrespective of whether the extract had been subjected to boiling or not. Similar intrapituitary infusions of synthetic arginine-vasopressin, adrenaline, histamine and other brain extracts, did not result in ovulation in 20 rats. Intravenous infusions of median-eminence extracts evoked ovulation only if an increased dose was administered. These results are therefore in good agreement with those presented above.

Since the first report in February 1960 of the present findings (see Harris, 1961), similar results have been published by two different groups of workers (McCann, Taleisnik & Friedman, 1960; McCann & Taleisnik, 1961; Courrier, Guillemin, Jutisz, Sakiz & Ascheim, 1961; McCann, 1962). McCann and his co-workers found that intravenous injection of an acid extract of pituitary stalk and median-eminence tissue of rats resulted in an increased concentration of LH in the plasma of recipient female rats, as determined with the Parlow assay method. Courrier et al. (1961) have injected acid extract of rat and sheep hypothalamic tissue into female rats and have observed an increased blood concentration of LH as revealed by the Parlow assay, and by the production of ovulation in anovular females. Neither of these groups obtained a similar response after injection of extracts of other brain regions, or of other control substances (such as vasopressin, oxytocin, histamine, adrenaline, serotonin). From these and other experiments both groups drew the conclusion that some substance present in their median eminence or hypothalamic extracts is active in stimulating the secretion of luteinizing hormone from the anterior pituitary gland.

Little is known as to the nature of the material, active in releasing LH, in extracts of the median eminence. It is stable when heated to 100° C for 10 min, and is reported (McCann, 1962) to be partially inactivated by pepsin and completely inactivated by trypsin.

The possibility therefore exists that it is a polypeptide. There is evidence that it is not vasopressin, vasotocin, oxytocin, adrenaline, histamine, serotonin, substance P, or (according to Courrier et al. 1961)  $\alpha$ - or  $\beta$ -melanophore-stimulating hormone, acetylcholine, bradykinin or noradrenaline.

From time to time suggestions have been made, based on more or less evidence, as to the nature of the substance responsible for the control of gonadotrophin secretion. Acetylcholine (Taubenhaus & Soskin, 1941), adrenaline (Markee, Everett & Sawyer, 1952), neurosecretory material of hypothalamic origin (Benoit & Assenmacher, 1953), posterior pituitary hormones (Shibusawa, Saito, Fukuda, Kawai, Yamada & Tomizawa, 1955; Desclin, 1956; Benson & Folley, 1957; Armstrong & Hansel, 1958; Martini, Mira, Pecile & Saito, 1959; Giuliani, Martini, Pecile & Fochi, 1961), and intermedin (Jöchle, 1956) have all been suggested in this respect. The studies described above, however, do not afford support for any of these suggestions.

### Summary

1. Infusions of extracts of the median eminence of the tuber cinereum, obtained from the brains of rabbits, cattle and monkeys, into the anterior pituitary gland of isolated female rabbits was found to excite secretion of gonadotrophic hormone (presumably LH) as shown by consequent ovulation in 55 out of 90 cases.
2. Similar infusions into the pituitary gland of control brain extracts (cerebral cortex, corpus callosum, caudate nucleus), and other naturally occurring substances (synthetic vasopressin, synthetic oxytocin, synthetic vasotocin, adrenaline, histamine, serotonin, substance P), resulted in ovulation in only 6 out of 95 cases.
3. Intravenous infusions of median-emminence extracts (in a dose of 20 times that of the minimal effective dose given by intrapituitary infusion, or more) was found to evoke ovulation in 6 out of 23 rabbits.
4. After consideration of the possible role played by direct damage to the pituitary gland, or of hormonal contamination of the infused extracts, the conclusion is drawn that the median eminence contains some substance which excites the secretion of LH from the anterior pituitary gland of rabbits. It seems likely that the release of this substance into the hypophysial portal vessels forms part of the neurohumoral mechanism controlling LH secretion.

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In many species marked sex differences in the control of endocrine function and behavior by the central nervous system (CNS) are an integral part of the reproductive process, including the recognition of a suitable sexual partner, in mating, and in the subsequent production and rearing of young. Sex differences in central nervous function represent the outcome of interactions between several different factors, among which the hormones secreted by the gonads are of paramount importance.

Current concepts of CNS sexual differentiation have their origins in a series of experiments, performed almost 50 years ago by Pfeiffer (1). His experiments with the laboratory rat showed that the expression of masculine patterns of pituitary gonadotropin secretion in adulthood depended on factors released from the testes during early postnatal life. Thus, the development of masculine patterns of gonadotropin secretion could be induced in genetic females by transplantation of a testis into the neck shortly after birth whereas castration of genetic males at birth resulted in the development of characteristically feminine patterns of gonadotropin release (1). With the later demonstration that the functions of pituitary are regulated by the hypothalamus, it became clear that the testes must influence the development of centers located within the brain (2).

Many other sexually differentiated neuroendocrine functions and behaviors are also dependent on early gonadal secretions. A general hypothesis has been formulated for the mechanism of CNS sexual differentiation which has much in common with the model for differentiation of the peripheral reproductive tract (3). The intrinsic pattern of CNS development is assumed to be organized along lines that are appropriate for the homogametic sex. In the heterogametic sex, differentiation away from this pattern occurs as a result of hormones produced by the gonads. Thus, in mammals the intrinsic pattern is female, with differentiation toward masculine patterns of gonadotropin secretion and behavior occurring in the male as a result of exposure to testicular hormones during development (4). In birds the homogametic sex is male, and differentiation of the

female CNS phenotype occurs as a result of exposure to ovarian hormones (5).

This simple mechanism is not the sole determining factor in sexual differentiation of the CNS. In many cases, however, there is good evidence that early hormonal experience makes at least some contribution.

#### Role of Early Gonadal Hormone Secretions

Although sex differences in CNS function exist in a great many vertebrate phyla, it is only in birds and mammals that these differences can be attributed to early gonadal hormone secretions. In fishes and amphibia, there are species in which early exposure to gonadal steroids induces complete sex reversal (6); but there is insufficient evidence to ascertain whether these effects involve a permanent developmental change in the CNS, or if they reflect primarily hormone-induced differentiation of peripheral structures.

The effects of early gonadal hormone secretions on mammalian and avian CNS function are extensive and diverse (table 9.1). In addition to reproductively oriented functions, such as sex behavior and the control of gonadotropin secretion, sex differences in a large number of other behavioral and neuroendocrine endpoints to some extent depend on early gonadal hormone secretions.

The diversity in the developmental effects of gonadal hormones raises the question of whether one or many different hormone-sensitive mechanisms are involved in CNS sexual differentiation. Although we cannot answer this question definitively, there is increasing evidence that separation of different developmental responses to gonadal hormones can occur. Species differ in the extent to which CNS functions are influenced by early gonadal hormone exposure. In rodents, early exposure to androgens from the developing testes results in permanent suppression of the capacity to support cyclic feminine patterns of gonadotropin secretion and instatement of the tonic release pattern characteristic of the male (2). There is no evidence for a similar early androgen-induced differentiation of the mechanism regulating pituitary function in the rhesus

**Table 9.1**

Some mammalian CNS functions are subject to organizational effects of early gonadal hormone exposure. Adapted from a more extensive summary of organizational and activational effects of gonadal hormones on nonreproductive behaviors in (97)

CNS Function	Animal	References
Regulation of gonadotropin, prolactin secretion	Rodents, sheep	(2, 4, 16)
Reproductive behaviors	Rodents, ferret, sheep, dog, rhesus	(4, 9, 17)
Nonreproductive behaviors		
Activity		
Running wheel	Rat	(86)
Open field	Rat, hamster	(87)
Intraspecies aggression	Rat, mouse	(88)
Play	Rat, rhesus monkey	(89)
Taste preferences	Rat	(90)
Scent marking	Gerbil	(91)
Feeding and body weight	Rat	(92)
Learning		
Active avoidance	Rat	(93)
Maze learning	Rat	(94)
Pituitary regulation of liver androgen metabolism via "feminotrophin" secretion	Rat	(95)
Circadian rhythms	Rat, hamster	(96, 97)
Response to brain lesions		
Septal area	Rat	(98)
Globus pallidus	Rat	(99)
Ventromedial hypothalamus	Rat	(100)
Orbital frontal cortex	Rhesus monkey	(101)

monkey or man (7, 8). Within species, some resolution of sexually differentiated CNS functions can be achieved. In mammals, it is now recognized that masculine CNS differentiation includes (i) suppression of the behavioral and neuroendocrine patterns characteristic of the female ("defeminization") and (ii) enhancement of the patterns characteristic of the male ("masculinization") (9). In a number of physiologic and pharmacologic conditions these processes can apparently occur independently (10).

How the effects of early gonadal hormone secretions on CNS development are expressed depends on several factors.

**1. Genetic factors** There may be differences between species in the neural substrate on which the hormones act, in terms of both hormone sensitivity and the function of specific hormone-sensitive structures. In the rat, afferent connections from structures outside the mediobasal hypothalamus (MBH) are essential for the maintenance of normal cyclic ovarian function (11). In the rhesus monkey, there is a lesser dependence on extra-hypothalamic input; thus complete deafferentation of the MBH does not abolish the capacity of female rhesus

monkeys to support cyclic patterns of gonadotropin release (8). Important genetic differences can also occur between members of the same species. In the extreme case, a genetic defect may result in complete loss of sensitivity to a particular gonadal hormone, as with the Tfm (testicular feminized male) mutation (3). More subtle gene effects may take the form of strain differences in sensitivity to hormones (12) or changes in the nature of the response to early hormone exposure (13).

**2. Hormonal effects in adulthood** These "activational" effects of the hormones differ from the earlier developmental or "organizational" effects in that they are not permanent but are reversed in the absence of hormones. In many animals, the expression of sexually differentiated reproductive behavior is absolutely dependent on appropriate circulating hormone levels. If the hormones are removed (for example, by gonadectomy), the behavior declines and can only be restored by replacement hormone therapy. Other sexually differentiated CNS functions vary in the extent to which they depend on the activational effects of gonadal hormones. There are, however, relatively few end points that are organized by gonadal hormones while remaining independent of later activational effects (10).

**3. Extrinsic influences from the environment and from social and learning experience** The impact of the environment is perhaps most obvious in species that have evolved seasonal breeding patterns. In such animals, hormone-induced sex differences in neuroendocrine function or behavior may be apparent only at certain points during the year, or under appropriate artificially controlled environmental conditions. In some birds, choice of an appropriate mate is strongly influenced by experiences that occur soon after hatching. Thus, cross-fostering of eggs from one species to parents of another may result in "sexual imprinting" of the young chicks, so that in later life their mating preference is directed toward the phenotype of the foster parent (14). In many mammals (including rats, guinea pigs, and rhesus monkeys) early social deprivation impairs subsequent masculine sexual behavior (15).

### Which Gonadal Hormones Are Involved?

The main testicular factor responsible for sexual differentiation of the mammalian CNS is probably testosterone, the major hormonal product of the developing testis. Treatment with testosterone can essentially substitute for the testis in masculinizing patterns of gonadotropin release and behavior (3, 9, 16, 17).

It would be premature, however, to conclude that sexual differentiation of the CNS is simply a function of the presence in males and absence from females of unbound circulating testosterone. Systematic radioimmunoassay measurements have shown that, in the lab-

oratory rat, androgens circulate in females as well as males during the period when sexual differentiation of the CNS is believed to occur. The amount of androgen present is still a matter of controversy: some reports have indicated consistently higher testosterone concentrations in males than in females during early postnatal life (18), whereas others have shown a considerable overlap in the amounts present in the two sexes (19). In a detailed study of plasma testosterone concentrations in rats killed between days 17 and 23 after conception (birth on day 21) Weisz and Ward found that only on day 18 were the values obtained from males consistently higher than those from females (20). In spite of the difference in results, these reports suggest that there may be significant levels of androgen in both sexes during early development. The partial data available for other mammalian species are consistent with the idea that, although there do appear to be periods during early development when circulating androgen levels are higher in males than in females, this difference is far from absolute and in many cases is not sustained (21).

The role of androgen in the female remains unclear. Brief exposure to high levels of testosterone may sensitize the developing male CNS to the effects of subsequent lower testosterone concentrations (20). Thus, sex differences in circulating testosterone might only be necessary when sexual differentiation begins: thereafter similar hormone concentrations could produce entirely different effects. There is evidence that low levels of androgen in female rodents at around the time of birth can lead to potentiation of feminine as well as masculine sexual behavior (10, 22). There may be hormonal factors in the female that serve to protect her from the differentiating effects of androgen. In rats the presence of the ovaries tends to inhibit the defeminizing effects of neonatal androgen treatment (23). Resko has suggested that in rhesus monkeys progesterone serves to protect developing female fetuses from circulating androgens, the major determinant of CNS differentiation being the circulating ratio of testosterone to progesterone, rather than simply testosterone concentrations (24). A similar mechanism has been proposed to operate in the rat (25), although controversy still exists over the question of whether there are sex differences in the progesterone content of serum from newborn rats (20).

Information regarding the hormones mediating avian sexual differentiation remains sparse. Avian embryos are capable of synthesizing estrogen (26); and in some birds this hormone seems likely to represent the ovarian product responsible for feminine differentiation. Administration of estrogen to developing male pigeons, chickens, or Japanese quail results in demasculinization of reproductive behavior patterns (5, 27); while

**Table 9.2**

Relation between the length of gestation and the timing of developmental critical periods for CNS sexual differentiation

Animal	Gestation or Incubation (Days)	Critical Period* (After Conception)
<i>Mammals</i>		
Rat	20 to 22	18 to 27 days
Mouse	19 to 20	Postnatal
Hamster	16	Postnatal
Guinea pig	63 to 70	30 to 37 days
Ferret	42	Postnatal
Dog	58 to 63	Prenatal + postnatal
Sheep	145 to 155	~30 to 90 days
Rhesus monkey	146 to 180	~40 to 60 days
<i>Birds</i>		
Japanese quail	17 to 18	Prehatching
Domestic chicken	22	Prehatching
Zebra finch	12 to 14	Posthatching
Pigeon	14	Posthatching

\* Figures are given only for those species in which the duration of the critical period has been systematically studied. For the other species listed, some doubt remains as to the precise timing of the period of CNS sensitivity to gonadal hormones. In these cases the information given (pre- or postnatal, pre- or posthatching) indicates when gonadal steroids have been shown to exert organizational effects on the CNS. The data are from (10, 102).

pharmacologic blockade of estrogen action in female quail prevents demasculinization (28). This pattern, however, is probably not common to all avian sexually differentiated behaviors. In the female zebra finch treatment with estrogen soon after hatching induces the development of male courtship and singing behaviors, but in males similar treatment is without effect (29).

### Developmental Periods of Hormone Sensitivity

In all species so far examined the CNS does not remain equally sensitive to the permanent organizational effects of gonadal hormones throughout early life. Instead, there is a developmental period for each species during which the CNS is more sensitive to these effects than at any other time. This period—commonly referred to as the “critical period” for sexual differentiation—has been delineated in four placental mammals (rat, guinea pig, sheep, and rhesus monkey) by examination of the effects of endogenous hormones and timed hormone treatments on sexually differentiated neuroendocrine and behavior patterns (table 9.2).

The critical period is an empirical concept, and does not represent a clearly defined stage of development. Nor does it necessarily encompass the entire period during which gonadal hormones contribute to the organization of CNS function. In practice, the end points commonly used to define the critical period are those associated with the control of reproductive function and sex behavior. It cannot be assumed that all

sexually differentiated CNS functions are maximally sensitive to gonadal hormones within the same period. Moreover, within the critical period itself there may be significant variability in the response of different end points to gonadal steroids. For example, in rats the mechanisms regulating cyclic secretion of gonadotropin and female sex behavior are most sensitive to androgen soon after birth and are relatively unaffected prenatally by androgen. Masculine sexual behavior, by contrast, is highly sensitive to androgen treatment before birth (10).

The available data suggest that the beginning of the critical period may follow differentiation of the testicular Leydig cells and the onset of testosterone secretion. In rats typical Leydig cells first appear between days 16 and 18 after conception, just before the presumed start of the critical period (30). In guinea pigs, testicular androgen production rises to a maximum at day 29 or 30 of gestation: in female guinea pigs organizational effects of prenatal androgen treatment first become apparent when the androgen is given on day 30 of gestation (31).

Morphological studies of the developing CNS have led to the hypothesis that maximal sensitivity to gonadal hormones may be associated with a particular stage of neuronal maturation. In the rat, many of the hypothalamic structures believed to be involved in sexual differentiation are poorly differentiated at birth (32). Studies on the effects of altered thyroid hormone status have shown that hyperthyroidism, which accelerates cerebral maturation, shortens the postnatal period during which the rat brain centers controlling gonadotropin secretion can be differentiated by treatment with low doses of androgen. In contrast, hypothyroidism prolongs this period (33). These results are consistent with the idea that the CNS may be most sensitive to androgen during some early phase of neural differentiation. A relation between the timing of the critical period and cerebral development may also exist in other species. Although the temporal relation between the critical period and the events of conception and birth is far from constant (table 9.2), this is at least in part a reflection of interspecies variability in the stage of development at which birth occurs. In species that are relatively less mature at birth (such as the rat, hamster, and pigeon), the critical period extends into postnatal life; whereas in animals that are more fully developed at birth (such as the guinea pig, rhesus monkey, and quail), the critical period tends to be predominantly or entirely prenatal (10).

Although CNS function can be organized by a single exposure to gonadal steroids during a critical period, differentiation is not always complete within this period. Full development of the response to early gonadal hormones may partly depend on subsequent hor-

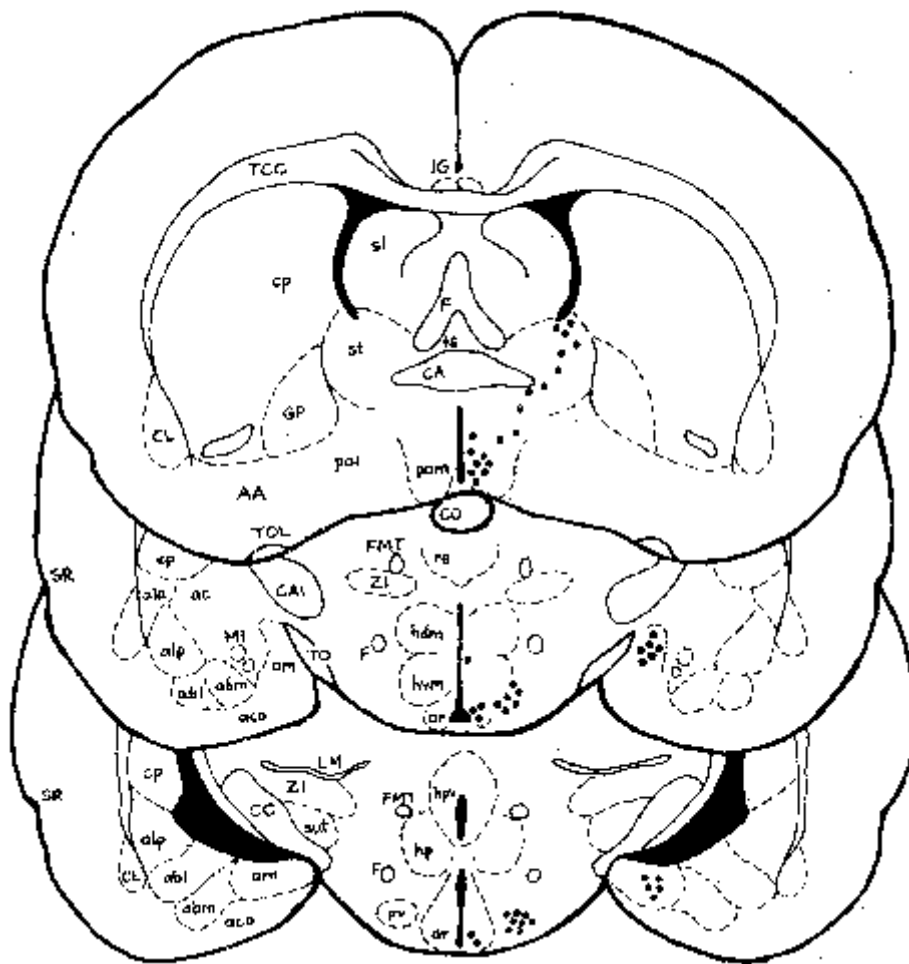
monal experience. For example, female rats treated neonatally with a low dose of androgen exhibit a few normal estrous cycles after puberty and then spontaneously become anovulatory (4, 34). This "delayed anovulatory syndrome" (DAS) depends on the continued presence of the ovaries. Thus, if the ovaries are removed prior to puberty the animal retains the capacity to support cyclic ovarian function well beyond the time at which it would normally be lost. These results suggest that complete development of sex differences in patterns of gonadotropin release may require the presence of gonadal steroids after the end of the critical period. Analogous findings have been reported for sexual behavior in the Japanese quail. Complete demasculinization of quail sexual behavior in the female is dependent on the presence of estrogen after hatching as well as in ovo (35).

### Hormone Target Cells in the Developing Brain

The effects of androgen on the CNS appear to be exerted directly, and do not require mediation by peripheral tissues. Thus, both anovulatory sterility and masculinization of patterns of sex behavior can be observed in female rats given intracranial testosterone implants soon after birth (36). The precise location of the androgen-sensitive sites within the brain, however, remains somewhat uncertain.

Two basic strategies have been followed in attempting to identify target areas for gonadal hormones in the developing CNS. First, the effects of stereotaxic hormone implants have been examined. In female rats, implantation of androgen into either the hypothalamus or the preoptic area shortly after birth results in a change in the pattern of subsequent sexual development (36). Christensen and Gorski (37) have reported that the responses elicited from these brain regions are dissimilar. The predominant effect of testosterone implants in the dorsal preoptic area is an increase in both masculine and feminine sex behaviors, but in the ventromedial hypothalamus similar implants inhibit both female sex behavior and the development of the capacity to support cyclic ovarian function.

The second strategy used to localize the effects of gonadal hormones is based on autoradiographic identification of sites of radiolabeled hormone concentration within the developing brain. Although there is no guarantee that sites of gonadal steroid uptake are necessarily involved in CNS differentiation, autoradiography has proved to be of considerable value in identifying hormone target cells. In the neonatal rat, Sheridan and co-workers (38) have identified within the hypothalamus, preoptic area, and amygdala several areas that concentrate  $^3\text{H}$ -labeled testosterone or its metabolites (figure 9.1). They observed strikingly similar distribu-



**Figure 9.1**

Topographic distribution of neurons that concentrate  $^3\text{H}$ -labeled testosterone or its metabolites in the newborn rat brain. The results of thaw-mount autoradiogram prepared from rats injected with  $^3\text{H}$ -labeled testosterone are presented schematically in coronal sections through the preoptic area, central hypothalamus, and central amygdala. Areas with dots on the right-hand side of the figure represent accumulations of radioactively labeled neurons. Abbreviations for labeled cell groups: *aco*, nucleus amygdaloideus corticalis; *am*, nucleus medialis amygdalae; *ar*, nucleus arcuatus; *hvp*, nucleus periventricularis hypothalami; *hvm*, nucleus ventromedialis hypothalami; *pom*, nucleus preopticus medialis; *pv*, nucleus pre-mammillaris ventralis; *st*, nucleus interstitialis striae terminalis. [From (38); courtesy of Karger]

tions of labeled cells after administration of  $^3\text{H}$ -labeled estradiol (38).

In the chick, Martinez-Vargas and coworkers have identified  $^3\text{H}$ -labeled estradiol-concentrating neurons in the preoptic area and hypothalamus on day 10 of incubation in ovo (39). By the time of hatching, other structures including the amygdala, ventrolateral septum, olfactory tubercle, and regions of the mesencephalon are also labeled by  $^3\text{H}$ -labeled estradiol.

#### Role of Androgen Metabolites: Aromatization Hypothesis

The importance of local metabolism in the mechanism of androgen action on peripheral target tissues is well documented (40). Figure 9.2 illustrates the major pathways by which potentially active metabolites of testosterone are formed within the neonatal rat brain. There

are essentially two pathways to be considered: the first involves  $5\alpha$ -reduction of the C4-C5 double bond to give  $5\alpha$ -dihydrotestosterone ( $5\alpha$ -DHT) which can then be further reduced at the 3 position to give  $3\alpha$ - and  $3\beta$ -androstenediol; the second involves aromatization of the A ring followed by hydroxylation at either the 2 or 4 position to yield the "catechol" estrogens (41).

Early exposure to estrogen affects mammalian feminine sexual development in much the same way as early exposure to testosterone. During the 1940's several workers reported that treatment of female rats with estrogen during gestation or shortly after birth resulted in a pattern of anovulatory sterility in adulthood which closely resembled that observed after perinatal testosterone administration (42). Subsequent work confirmed and extended these findings; many of the effects of testosterone on the developing brain were

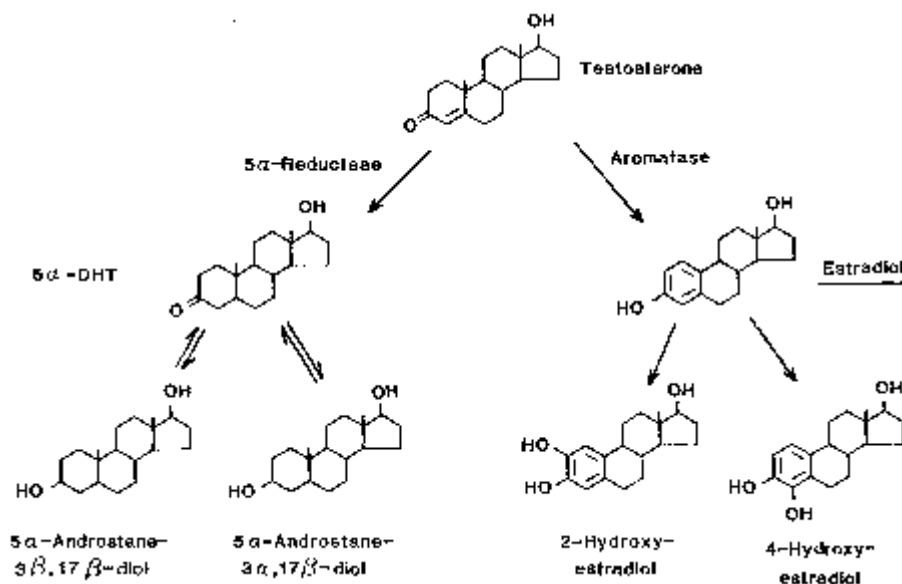


Figure 9.2

Major possible routes of testosterone metabolism in developing brain tissue which result in the formation of physiologically active steroids (10, 41, 46).

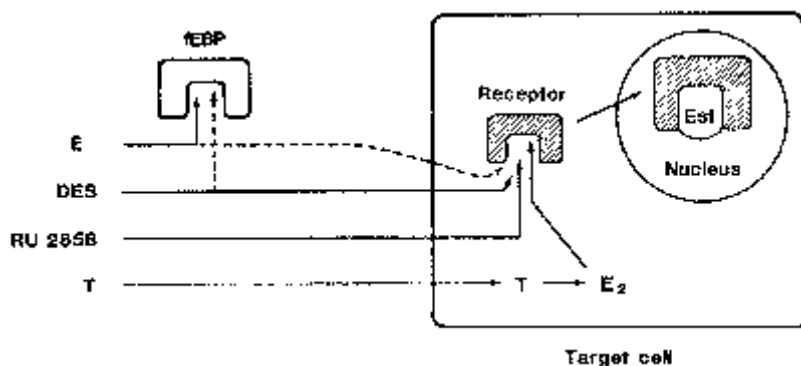
produced by estrogen (43). The full significance of these observations did not become apparent, however, until the early 1970's, when three observations brought about a complete reevaluation of the role of estrogen in sexual differentiation. First, 5 $\alpha$ -DHT and other 5 $\alpha$ -reduced androgens were shown to be far less effective than either testosterone or estradiol at inducing defeminization of the neonatal rat brain (44). Second, the effects of testosterone treatment in neonatal female rats could be blocked by administration of the estrogen antagonist MER25 (45). Third, the developing brain itself is a site of androgen-to-estrogen conversion (46). These findings indicated that estrogen formation might play an important part in mediating the developmental effects of testosterone.

We now know that, in the rat, local estrogen formation plays a crucial role in sexual differentiation of the brain. Selective pharmacologic inhibition of estrogen formation from androgen or of the interaction between estrogen and its receptor sites dramatically impairs the developmental response of the CNS to perinatal testosterone (47). Studies with synthetic estrogens have established that the developing rat brain is exquisitely estrogen-sensitive: the minimum dosage required to produce defeminization of behavior and gonadotropin secretion in neonatal female rats is considerably lower for estrogens than for testosterone. Particularly compelling evidence for the role of aromatization has come from studies of Tfm male rats, which have severely reduced levels of androgen receptors compared to their normal siblings but show normal CNS levels of both estrogen receptors and aromatase (48). If androgen-

specific receptor mechanisms played an indispensable role in the actions of testosterone on the developing brain, Tfm male rats would not be expected to undergo brain sexual differentiation. In fact, this does not appear to be the case. Patterns of gonadotropin release and sex behavior in Tfm male rats do differentiate under the influence of testicular secretions (49).

It would be premature, however, to conclude that testosterone and the 5 $\alpha$ -reduced androgens play no direct role in CNS sexual differentiation. Several observations seem inconsistent with the idea that androgen acts entirely via conversion to estrogen. Testosterone-induced sexual differentiation is inhibited by androgen antagonists as well as estrogen antagonists (50). In male rats castrated at birth the developmental effects of systemic low-dose estrogen treatment seem to be potentiated by the simultaneous administration of 5 $\alpha$ -DHT (51). Although sex behavior in Tfm male rats is clearly differentiated toward the masculine phenotype, the saccharin preference of these males remains female (52).

In other mammalian species there is a good deal of variability in the extent to which estrogen is required for sexual differentiation of the CNS. In female guinea pigs and rhesus monkeys, the expression of masculine sex behavior can be potentiated by prenatal treatment with 5 $\alpha$ -DHT. Suppression of female sex behavior and cyclic ovarian function in guinea pigs, by contrast, may involve aromatizable androgens (10). The hamster seems very much like the rat in that both the masculinization and defeminization aspects of sexual differentiation can be induced by either aromatizable androgens or estrogen (53, 54).



**Figure 9.3**

Schematic diagram of the protective role of fetoneonatal estrogen binding protein (*fEBP*) in neonatal rats, and the ability of synthetic estrogens and testosterone to bypass this mechanism. Abbreviations:  $E_2$ , estradiol; *DES*, diethylstilbestrol; *Ru2858*, 11 $\beta$ -methoxy-17 $\alpha$ -ethynylestradiol; *T*, testosterone; *Est*, nuclear bound estrogens. [From (68); courtesy of *Brain Research*]

The role of the other androgen and estrogen metabolites remains to be established. The effects of the 3 $\alpha$ - and 3 $\beta$ -androstane diols are difficult to investigate critically, in view of the possibility of back conversion of these compounds to 5 $\alpha$ -DHT. With regard to the catechol estrogens, we do know that both the 2- and 4-hydroxy estrogen derivatives are capable of defeminizing patterns of gonadotropin release when injected into neonatal female rats [4-hydroxyestradiol being at least as potent as estradiol with regard to this end point (55)]. It remains uncertain, however, whether this indicates any *specific* involvement of catechol estrogens in sexual differentiation.

#### $\alpha$ -Fetoprotein and the Protection Hypothesis

In placental mammals, the fetus is continually exposed to endogenous estrogen from the placenta and maternal circulation. If estrogen formation within the brain plays a vital role in sexual differentiation, then it follows that the fetus must somehow be protected from the effects of circulating estrogen. In rats and mice, the mechanism by which this protection is achieved is well established. In these two species, the immature brain is functionally protected from circulating estrogen by a plasma estrogen binding system. The developing yolk sac and fetal liver synthesize an estrogen binding protein (fetoneonatal estrogen binding protein, or *fEBP*) which circulates at high concentrations during the latter part of gestation and then gradually disappears over the first few weeks of postnatal life (56). This protein, which is immunochemically indistinguishable from the plasma  $\alpha$ -globulin,  $\alpha$ -fetoprotein, binds and effectively sequesters much of the estrogen present in the fetal and neonatal circulations. Significantly, however, it does not bind testosterone: hence testosterone is free to enter the brain where it can be converted to estrogen and interact with cellular estrogen receptors (figure 9.3).

Several experimental observations confirm the effectiveness of the *fEBP* protection mechanism. Although the levels of estradiol in the blood of the neonatal female rat are high, free estradiol does not seem to be readily available in the tissues (57). The administration of antibodies to  $\alpha$ -fetoprotein newborn female rats produces effects on sexual development that resemble those of estradiol injections (58). Particularly compelling evidence has come from studies with synthetic estrogens. As indicated in figure 9.3, synthetic estrogens such as diethylstilbestrol (*DES*) and *Ru 2858* exhibit markedly lower affinities than estradiol for *fEBP*. As might be expected if *fEBP* does indeed serve to protect the developing tissues, *DES* and *Ru 2858* are more potent than estradiol in inducing sexual differentiation of female rat brain (59).

It is not yet possible to extend the *fEBP* protection hypothesis to species other than the rat and mouse. Although  $\alpha$ -fetoprotein is present in many developing vertebrates, the ability to bind estrogen seems to be a property of a particular  $\alpha$ -fetoprotein variant. Indirect evidence, based on the relative potencies of estradiol and *Ru 2858*, suggests that the situation in the hamster may be similar to that in the rat (54). In contrast, in humans only a small proportion ( $\sim 0.1$  percent) of circulating  $\alpha$ -fetoprotein binds estrogen (60), while in guinea pigs attempts to demonstrate a specific fetal blood-borne estrogen binding system have so far been unsuccessful (61).

However, elements of the aromatization-estrogen response mechanism are clearly present in guinea pig and human fetuses. As was mentioned earlier, guinea pig sexual development is sensitive to prenatal exposure to estrogens, as well as androgens. The human fetal brain is capable of converting androgen to estrogen (46). There is no *a priori* reason to suppose that aromatization does not play a part in the response of the developing human or guinea pig CNS to prenatal androgen.

Yet, in both man and guinea pig, we apparently cannot invoke  $\alpha$ -fetoprotein as a mechanism for the regulation of free circulating estrogen levels during gestation.

The cellular localization of  $\alpha$ -fetoprotein within the rodent brain has been studied.  $\alpha$ -Fetoprotein is present in the neonatal rat brain as well as in the bloodstream (62). It is not merely an extracellular component of immature brain tissue, but is in part localized within developing nerve cells. This localization exhibits a striking regional specificity: those regions of the hypothalamus, preoptic area, and amygdala that concentrate  $^3\text{H}$ -labeled testosterone and  $^3\text{H}$ -labeled estradiol from the circulation are apparently devoid of intracellular immunoreactive  $\alpha$ -fetoprotein (63). These observations suggest that the rat and mouse FEBP- $\alpha$ -fetoprotein systems may have more than just a protective role—possibly serving as a modulator of intracellular estrogen rather than as a strict barrier to entry of the hormone (32, 63).

### Steroid Receptors in the Developing Brain

The general model proposed for steroid hormone action involves an initial binding reaction between the hormone and a stereospecific cytoplasmic receptor site. Subsequently, the hormone-receptor complex moves to the cell nucleus, where it is bound to the chromatin, initiating a cellular response (3, 40). This type of receptor system is believed to mediate many of the activation effects of steroid hormones on CNS function (64). We now believe that the organizational effects of estrogens and aromatizable androgens on the developing rat brain are mediated through a similar receptor mechanism (figure 9.3).

Much of the evidence for this stems from studies in the rat. Putative cytoplasmic receptors for androgens, estrogens, and progestins have now been identified in brain extracts of the newborn rat (65, 66). Physicochemically, the receptors appear similar to the homologous receptors from mature brain tissue. Two factors, however, set the neonatal systems apart from those present in adulthood. (i) The tissue concentrations of receptor sites are not static, but change rapidly both during and after the perinatal critical period; (ii) the regional distribution of estrogen receptors within the brain changes during development. In adulthood, these receptors are concentrated in structures within the corticomedial amygdala, preoptic area, and medio-basal hypothalamus. In neonatal rats, however, a diffuse population of cells containing estrogen receptors is also found extending through layers 5 and 6 of the cerebral cortex (67). These cortical estrogen receptors decline to adult levels after postnatal day 10 (68, 69).

Autoradiographic and biochemical studies indicate that the androgen and estrogen can bind within cell nuclei in the developing rat brain. The biochemical data show that the estrogen binding systems are capable of interacting with estradiol synthesized locally from androgen. After the administration of  $^3\text{H}$ -labeled testosterone to newborn rats, much of the radioactivity recovered from amygdaloid, preoptic area, and hypothalamic cell nuclei represents  $^3\text{H}$ -labeled estradiol (although some labeled testosterone and  $5\alpha$ -DHT is retained) (66). Studies on the extent to which brain estrogen receptors are translocated into cell nuclei by endogenous gonadal steroids in newborn rats show higher cell nuclear receptor concentrations in males than in females. This sex difference is abolished by castration, or treatment with the aromatase inhibitor ATD (1,4,6-androstatriene-3,17-dione) (70).

It seems likely that similar receptor systems are present in other species during early development. Cytoplasmic androgen and estrogen receptors have been identified in the mouse brain during perinatal life (71). Similarly, putative estrogen receptors have been identified in cytoplasmic fractions prepared from the brains of fetal guinea pigs (61). Autoradiographic studies (39) suggest that functional CNS estrogen receptor systems are probably present in the chick embryo well before hatching.

### Biochemical Effects of Early Hormone Exposure

The cellular mechanisms that translate early exposure to gonadal steroids into a permanent developmental effect remain ill-defined. For the most part, what we know is based on indirect or circumstantial evidence. An important clue is provided by the similarity between the steroid binding systems present in the adult and developing brain. In mature tissues, the first step in the mechanism of response to steroid hormones is thought to be the initiation of changes in nucleic acid and protein synthesis (3, 40). It seems reasonable to suppose that in the developing brain cell nuclear binding of steroid-receptor complexes may initiate similar biochemical events.

There is some evidence to support this hypothesis. In female rats, antibiotic inhibitors of protein and nucleic synthesis afford a degree of protection against the differentiating effects of early androgen injections (72). General effects of androgen on protein and RNA metabolism in the developing rat brain have been reported (73). It remains to be seen whether these effects reflect specific changes in cerebral macromolecular synthesis associated with sexual differentiation.

In the adult rodent brain an important consequence of gonadal steroid exposure is altered neurotransmitter

function (64). There is some evidence to suggest that similar mechanisms may also operate during development. In 12-day-old rats, brain serotonin concentrations are higher in males than in females (74). This difference appears to be the result of perinatal androgen secretions. Several drugs that interfere with monoaminergic transmission (chlorpromazine, pargyline, reserpine) and an inhibitor of acetylcholinesterase (pyridostigmine) also influence sexual differentiation (75).

Insight into the way in which steroid effects on the developing CNS may ultimately be expressed has been provided by Toran-Allerand (76), who showed, using an in vitro culture technique, that estradiol and testosterone accelerate and enhance the outgrowth of neurites from explants of newborn mouse preoptic area and infundibular and premammillary regions of the hypothalamus. The effect is region-specific, other regions showing little or no response in culture to the same hormonal treatments. The hormonal specificity of the response is similar to that of androgen-induced CNS sexual differentiation. In contrast to testosterone, 5 $\alpha$ -DHT has no measurable effect on neurite outgrowth in culture. Estrogen appears to be of primary importance: thus, the neuritic response can be blocked by adding either antibodies to estradiol, or the synthetic estrogen antagonist C1628 to the culture medium. These results suggest that the effects of estrogens and aromatizable androgens on the developing brain may involve a regional stimulation of neurite growth. This hypothesis assumes particular importance in view of the emerging evidence that early gonadal hormone exposure may actually induce structural alterations within the CNS.

### Morphologic Sex Differences in the CNS

As table 9.3 indicates, there is a growing list of examples of sex differences in CNS morphology. These differences fall into three general categories: (i) ultrastructural differences in cellular or synaptic organelles, (ii) differences in synaptic or dendritic organization, and (iii) differences in the gross volume of defined cell groups (figure 9.4). Many of these sex dimorphisms are at least partly dependent on early gonadal hormone secretions (32).

With two exceptions, neuroanatomic differences cannot be correlated with specific sexually differentiated CNS functions.

1. In the zebra finch (*Poephila guttata*) the avian song control system consists of a chain of five discrete brain nuclei, which are involved in the efferent motor pathway for singing behavior (77). In some birds the

**Table 9.3**

Androgen-dependent sexual dimorphism in the CNS. The data in this table are expanded from a similar summary in (32)

Sex Difference	Region	Animal	References
Neuronal nuclear and nucleolar size	Preoptic area, amygdala, ventromedial hypothalamus	Rat	(103)
Synaptic vesicles and terminals	Arcuate	Rat	(104)
Synaptic organization	Preoptic area	Rat	(105)
Dendritic branching patterns	Preoptic area Suprachiasmatic nucleus Hippocampus (Ammon's horn)	Hamster, rat Mouse	(105) (106)
Gross nuclear volume	Preoptic area Lumbar spinal cord (5th and 6th segments) Nucleus robustus archistriatalis; nucleus hyperstriatum ventrale pars caudalis	Rat Rat Zebra finch	(107) (77) (78)

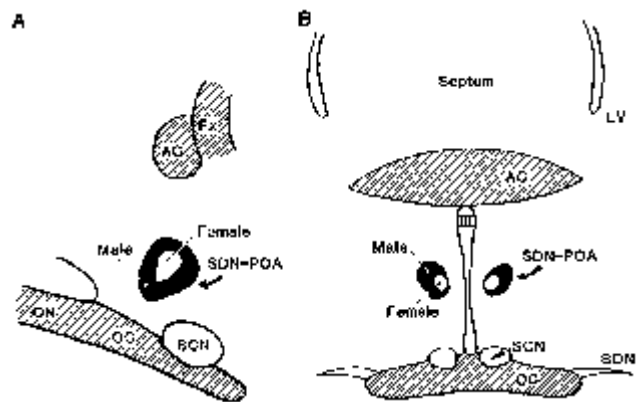
expression of male-like song depends on testicular androgen to the extent that castration inhibits and testosterone replacement restores male song patterns. Male singing is a function of gonadal hormone secretions soon after hatching, as well as androgen in adulthood. It has been shown that the CNS song control centers are larger in male zebra finches than in females (77); and for two of these centers the nucleus robustus archistriatalis (RA) and the nucleus hyperstriatum ventrale pars caudalis (HVC), exposure to estrogen soon after hatching is required for development to occur along masculine lines (29). Estrogen treatment of newly hatched female finches results in a significant increase in the volumes of these two nuclei and, in addition, potentiates the response of the RA and HVC to androgens given in adulthood. These anatomic changes are paralleled by behavioral sensitivity to androgen such that females treated neonatally with estrogen show patterns of courtship and song behaviors in response to adult androgen treatment that closely resemble those of the normal male.

2. In male rats, testicular androgen released around the time of birth differentiates the complex of androgen-dependent penile spinal reflexes that are characteristic of male sexual behavior (78). In the spinal cords of male rats, a discrete group of androgen-concentrating motoneurons innervating the levator ani (LA) and bulbocavernosus (B) penile striated muscles have been identified and termed the spinal nucleus of the bulbocavernosus (SNB) (79). But in female rats, the LA and B muscles are either absent or vestigial; and the volume of the SNB is greatly reduced (79).

### Mechanisms Involved in the Genesis of Morphologic Sex Differences

The mechanisms involved in the development of sex differences in CNS morphology remain unknown. Given the diversity of the structural differences which have been described, it is difficult to formulate a generally applicable hypothesis. One attractive possibility is that anatomic sex differences may represent an expression of growth-promoting effects of gonadal steroids, analogous to those observed in culture by Toran-Aillerand (76). Acceleration and enhancement of the development of one component of the neural circuits involved in regulating reproductive function could have far-reaching consequences in terms of the differentiation of postsynaptic cellular and dendritic component and competition for available postsynaptic space, as well as the specific formation of new synaptic contacts (10, 32). Accelerated growth might bring about the survival of neural elements that would otherwise be eliminated competitively during later life. Several lines of evidence suggest that the survival of neurons may to some extent be dependent on the formation of synaptic contacts, cells that form from only a limited number of synaptic connections being preferentially eliminated during CNS maturation (80). Moreover, some loss of synaptic connections appears to be a normal feature of CNS differentiation (81). Gonadal steroids could alter this process, by selectively stabilizing some connections or enhancing the rate of degradation of others (or both).

Processes of enhanced growth and degeneration are not necessarily confined to early development. They may simply be more obvious during early life, as a result of the greater plasticity of the immature CNS. Thus, under some circumstances morphologic effects of gonadal steroid treatment can be demonstrated in the adult animal. Arai and coworkers have reported that estrogen treatment of neonatal female rats has a marked synaptogenic effect with respect to the formation of axodendritic synapses in the arcuate nucleus (82). This effect is not observed when estrogen is given in adulthood. If, however, the mediobasal hypothalamus is deafferented in adulthood (resulting in degeneration of a proportion of the presynaptic elements in the arcuate nucleus), the ability of estrogen to stimulate axodendritic synapse formation is restored. A similar effect may underlie reports that the behavioral response of adult male rats to septal lesions can be modified by postoperative treatment with estrogen (83). With respect to degenerative effects, estrogen treatment of adult female rats results within a few months in marked neuropathologic changes in the arcuate nucleus and loss of ovarian cyclicity (84). This may represent an exaggeration of a normal gonadal



**Figure 9.4**

Schematic localization of the sexually dimorphic component of the medial preoptic nucleus (*SDN-POA*) of the rat brain in the sagittal (A) and coronal (B) planes. *AC*, anterior commissure; *Fx*, fornix; *LV*, lateral ventricle; *OC*, optic chiasm; *ON*, optic nerve; *SCN*, supra-chiasmatic nucleus; *SON*, supraoptic nucleus; *III*, third ventricle. [(107); courtesy of *Brain Research*]

steroid-induced aging process: similar neuropathologic changes are observed in the arcuate nucleus of old (12 months), spontaneously acyclic rats; but, if ovariectomy is performed at 2 months of age the extent of the neuropathologic change at 12 months is reduced (84).

Our understanding of the way in which morphologic changes in the CNS are brought about would be greatly facilitated if a single defined group of sexually dimorphic neurons could be followed throughout development. This may soon become possible (85). Gorski et al. have described the development of the sexually dimorphic nucleus of the medial preoptic area of the rat (*SDN-POA*) (figure 9.4) from gestation through to adulthood (85). An increase in the size and number of neurons within the nucleus is apparent in the male starting at around the time of birth and continuing throughout the first 10 days of postnatal life. Interestingly, thymidine-labeled autoradiographic studies have shown that a small proportion of *SDN-POA* neuroblasts undergo final cell division as late as day 18 of gestation, when sex differences in circulating androgen are clearly apparent (20). Thus, it seems theoretically possible to specifically label neurons within the *SDN-POA* at the time of their final cell division and follow the morphologic effects of gonadal steroids on these cells into later life.

### Conclusions

In the majority of species that have been studied the experimental data are consistent with the basic hypothesis for the mechanism of CNS sexual differentiation described. The hormonal products of the heterogametic gonad are of fundamental importance to the development of sex differences in CNS function. Thus,

if the action of these hormones is prevented, sexual differentiation of the CNS is impaired.

This emphasis on the role of the heterogametic gonad does not necessarily mean that CNS development in the homogametic sex is entirely passive and independent of hormonal effects. Although the heterogametic sex in birds is female, song and courtship patterns in the zebra finch apparently differentiate in the male in response to early gonadal secretions (29). In mammals, the hormones released by the testis are important for masculine sexual differentiation; but this does not preclude the possibility that hormones may contribute to feminine CNS development. Factors such as progesterone may actively protect the female fetus from the influence of circulating androgen. Moreover, low concentrations of androgen or estrogen may actively promote the development of feminine behavioral traits.

The way in which gonadal steroids exert their effects on the developing CNS is not yet fully understood. Certain common features have emerged. Sensitivity to the differentiating effects of the hormones is high during early development. In many species, estrogen plays at least some part as either a circulating hormone or a locally active metabolite of circulating androgen. The initial step in the response mechanism may well involve a binding reaction between the hormones and cellular receptors, which closely resemble the receptors present in other steroid target tissues. Ultimately, the effects of early hormone exposure are expressed in terms of changes in the CNS at the structural as well as functional level.

A major unanswered question is how the initial reaction between the hormones and the developing CNS is translated into a permanent differentiating effect. Circumstantial evidence suggests that a hormone-induced change in gene expression may be involved, perhaps resulting in a cellular growth response. However, we really cannot be certain that this is the mechanism by which sexual differentiation occurs. For the most part, it remains impossible even to state where in the CNS the hormones act to bring about differentiation of particular behavioral and neuroendocrine functions, let alone to determine how these effects are produced. The introduction of in vitro and in vivo model systems for examining the response of specific cell groups to early gonadal hormone exposure offers valuable new approaches to this important remaining problem.

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The mammalian Y chromosome plays a crucial part in sex determination: an embryo that inherits a Y chromosome develops as a male whereas an embryo lacking a Y chromosome develops as a female (1). The sex determining gene(s) on the Y chromosome induces testicular development, and subsequent male sexual differentiation is a consequence of the hormonal products of the testis (2). The Y-encoded testis-determining gene has been named TDF (testis-determining factor) in humans and *Tdy* (testis-determining Y chromosome) in mouse. Although it is likely that many different genes are required for both male and female sex determination, understanding the mode of action of TDF may provide a general model for the genetic control of developmental decisions in mammals.

Attempts to identify and clone TDF have exploited detailed maps of the Y chromosome. Three types of map have been constructed: deletion maps from analysis of the genomes of sex-reversed XX males and XY females (3, 4); a meiotic map of the pseudoautosomal region which is shared by the X and Y chromosomes (5, 6) and a long-range restriction map linking the first two maps (7). Most XX males have inherited Y-derived sequences, including TDF, by terminal exchange between the X and Y chromosomes (8, 9). A map constructed on the basis of the Y fragments present in different XX males placed TDF in the distal part of the Y chromosome adjacent to the pseudoautosomal region (3). The meiotic map of the pseudoautosomal region indicated that MIC2 was the closest known pseudoautosomal locus to the sex-specific part of the Y chromosome (5). A long-range restriction map beginning at MIC2 and extending into the sex-specific region of the X and Y chromosomes identified a CpG-rich island on the Y chromosome which represented a possible candidate gene for TDF (7). This gene was cloned after a chromosome walk initiated 130-kilobase (kb) proximal to the CpG-rich region, and subsequently named ZFY (10). The sequences present in a particular XX male (LGL203) and absent in an XY female (WHT1013) were used to define the position of TDF to within an interval of 140 kb and ZFY was isolated from this region. Other evidence consis-

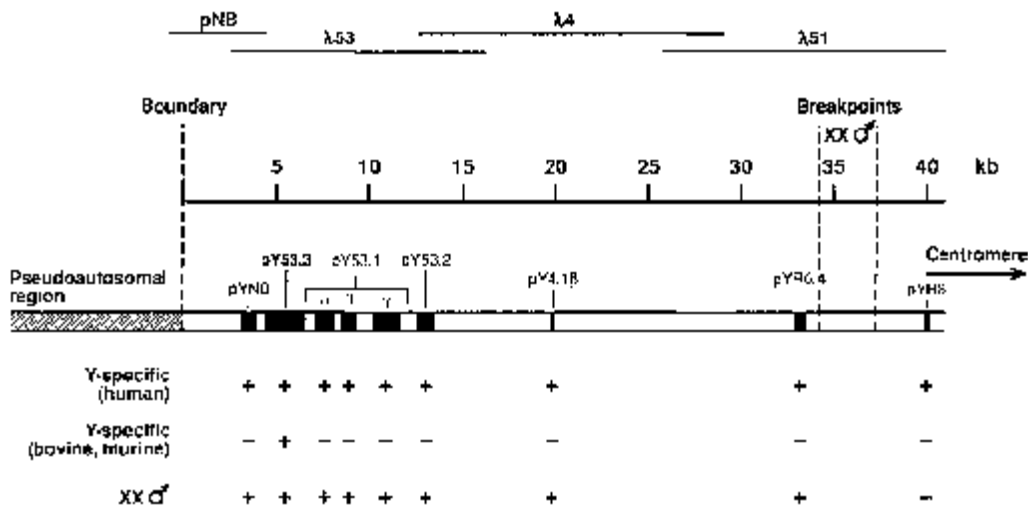
tent with identity between ZFY and TDF included the finding of ZFY-related sequences on the Y chromosome of all eutherian mammals tested; the presence of a ZFY-related gene. *Zfy-1*, in *Sxr'* (the smallest part of the mouse Y chromosome known to be sex-determining) and the structure of the ZFY-encoded protein, which has many features in common with transcription factors (10, 11). But there were several unexpected findings; first, ZFX—a homologue of ZFY—was found on the eutherian X chromosome and shown in humans to escape inactivation (11, 12), and second, in metatherian mammals (marsupials), ZFY-related sequences were found not on the Y or X chromosome, but on the autosomes (13).

Two recent reports have further questioned the role of ZFY in male sex-determination. Koopman et al. studied the expression of *Zfy-1* and *Zfy-2*, the mouse homologues of ZFY, and found that their expression was linked to germ cells—a cell-type not required for normal male development. Furthermore, in *W<sup>e</sup>/W<sup>e</sup>* mutant mice, testicular development occurred in the absence of detectable *Zfy-1* and *Zfy-2* expression (14). Palmer et al. described four sex-reversed XX individuals that had inherited Y-derived sequences not including ZFY. Assuming that these individuals are male because of their Y-derived sequences, this mapped TDF to the 60 kb proximal to the pseudoautosomal boundary. This result is inconsistent with the published breakpoint of the XY female (WHT1013) (10) and formally excludes ZFY as a candidate for TDF (15).

We have tested the hypothesis that the region immediately adjacent to the boundary contains TDF by searching for a gene in this region. We have found an open reading frame that is part of a conserved, Y-specific gene. This gene also shares homology with the *Mc* gene of *Schizo saccharomyces pombe* and with a conserved DNA-binding motif present in non-histone proteins related to HMG1 and HMG2.

#### Analysis of 35 kb of the Y Chromosome

We have previously described a chromosome walk, comprising a series of overlapping  $\lambda$  and cosmid clones,

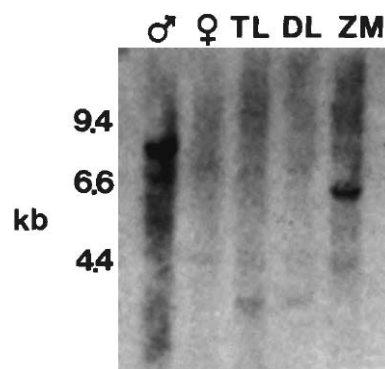


**Figure 10.1**

Map of distal short arm of human Y chromosome: shaded region (left), pseudoautosomal region; broken line, boundary between pseudoautosomal and Y-specific regions. Top, the three overlapping lambda clones  $\lambda 51$ ,  $\lambda 4$ ,  $\lambda 53$  and the plasmid pNB obtained from walking along the Y chromosome. Breakpoints of the XX males, broken lines at 35 kb (see figure 10.2). Black boxes, probes detecting single-copy Y-specific human DNA fragments (+). When these probes were hybridized to male and female DNA from bovine and murine genomes, only pY53.3 detected Y-specific fragments (+). All of the probes except pYH8 hybridized to sequences in the XX males. pY53.3 was known as H2.1 and pY53.2 was called P0.9 (ref. 16).

from the pseudoautosomal region, across the pseudoautosomal boundary, to the sex-specific region of the Y-chromosome (16). Probes from this walk were used by Palmer et al. on the genomes of XX males to define the region in which TDF must lie (15). Since then, three additional probes have been used on the genomes of the same ZFY-negative XX males: pY4.1 $\beta$ , which was positive with the XX males, and pYH8, which was negative (figure 10.1). The third Y-specific probe, pYR0.4, is derived from sequences lying between pY4.1 $\beta$  and pYH8, and seems to define the breakpoints in the XX males. The probe pYR0.4 detects an 8.5-kb *Hind*III fragment in normal males but only a 4-kb fragment in two related individuals, an XX male and an hermaphrodite; a 6-kb fragment was detected in a third XX male (figure 10.2). The breakpoints in the XX males are clustered around a region that is roughly 35 kb proximal to the boundary (figure 10.1). This result implies that *TDF* is located in sex-specific sequences within 35 kb of the pseudoautosomal boundary. Further refinement of the positions of the breakpoints was not possible because of the highly repetitive nature of the sequences between pY4.1 $\beta$ , pYR0.4 and pYH8.

The following strategy was adopted to locate *TDF* within this 35-kb region of the Y chromosome. DNA from this region was subcloned into fragments of about 4 kb in size. Subsequently, each 4-kb fragment was cleaved with frequently cutting restriction enzymes such as *Rsa*I, to produce smaller fragments in the size range 500 base pairs (bp) to 1 kb. A total of 50 probes



**Figure 10.2**

Southern blot analysis of *Hind*III-digested genomic DNA from: ♂, 46XY cell line, PGF (ref. 28); ♀, 46XX cell line, WT49 (ref. 29); TL, familial cases (XX male); DL (hermaphrodite) and ZM (unrelated XX male) hybridized with pYR0.4. The probe detects an 8.5-kb *Hind*III fragment in normal males but only a 4-kb fragment in the two related individuals TL and DL, and a 6-kb fragment in ZM. This implies that breakpoints in these XX males and hermaphrodite are clustered around a region 35 kb from the boundary (see figure 10.1). DNA size markers (kb) to the left.

**METHODS.** Genomic DNA (10  $\mu$ g) was digested with *Hind*III, separated on a 0.8% agarose gel, transferred to Hybond N<sup>+</sup> (Amersham) and fixed in 0.4 M NaOH (20 min). To suppress repeat sequences present in the probe pYR0.4 it was labelled with <sup>32</sup>P, denatured together with 500  $\mu$ l (5 mg ml<sup>-1</sup>) sonicated human placental DNA and prehybridized in 2 ml hybridization buffer at 65 °C for 2 h<sup>17</sup>. This probe mixture (2 ml) was added to the filter in a 5  $\times$  SSPE buffer, 5  $\times$  Denhardt's solution, 0.5% sodium dodecylsulphate, 200  $\mu$ g ml<sup>-1</sup> denatured salmon-sperm DNA and 10% sonicated denatured human placental DNA, and hybridized for 16 h at 65 °C. The filter was extensively washed with 0.2  $\times$  SSC buffer, 0.2% SDS at 65 °C and autoradiographed for 6 days.

generated in this manner were examined. Each small fragment was radioactively labelled and used to probe Southern blots of DNA from human males and females, murine males and females, bovine males and females and human–hamster somatic cell hybrids containing the human X or the human Y chromosome. All probes were tested with and without prehybridization to total human DNA to suppress the contribution of repetitive sequences (17). Despite this latter precaution, most probes tested failed to detect unique sequences in the genome of humans but reacted with repetitive elements distributed throughout the genome. These repetitive probes frequently also detected repeat sequences in the bovine genome.

Seven probes were found that detect single-copy Y-specific bands in *Eco*RI-digested human DNA (figure 10.1); pYNB (0.7 kb), pY53.3 (2.1 kb), pY53.1 $\alpha$  (0.8 kb), pY53.1 $\beta$  (0.8 kb), pY53.1 $\gamma$  (1.3 kb), pY53.2 (0.9 kb) and pY4.1 $\beta$  (0.2 kb). But of the seven probes, only pY53.3 also reacted with Y-specific bands in the murine and bovine genomes. A 0.9-kb *Hinc*II subclone of pY53.3 hybridized most strongly to Y-specific fragments in human, murine and bovine genomic DNA. This subclone was used as a probe in subsequent experiments.

### Conservation of pY53.3

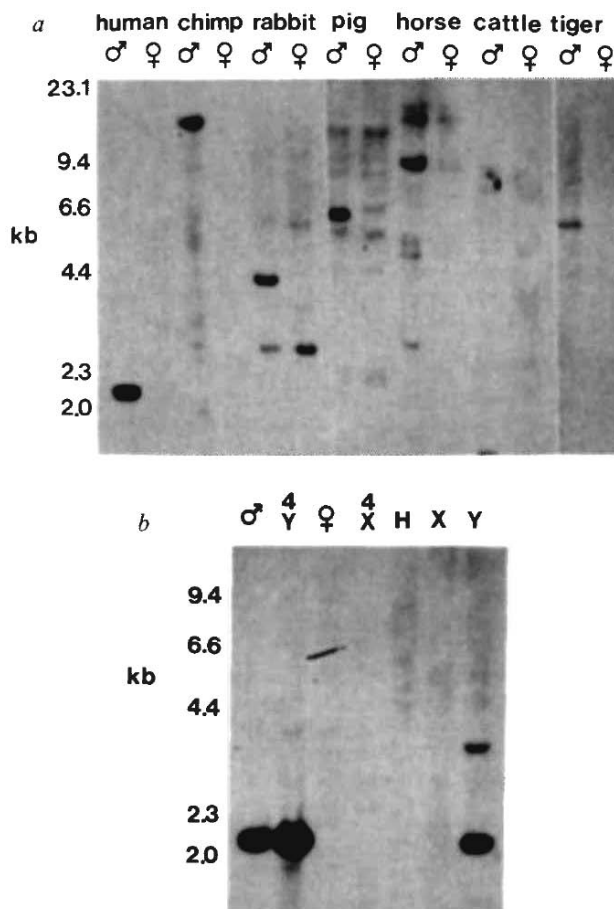
A “Noah’s Ark” blot containing DNA from male and female pairs of eutherian mammals was hybridized with the 0.9-kb *Hinc*II subclone of pY53.3 (figure 10.3a).

The sequences detected by this probe are conserved and male-specific in a wide spectrum of eutherian mammals. At low stringency, additional fragments were found that are shared between males and females, suggesting the existence of pY53.3-related X-linked or autosomal sequences. But these fragments were not detected in a human–rodent somatic cell hybrid that retained the human X chromosome as the only human contribution (figure 10.3b).

Unique sequences that are conserved between the Y chromosomes of different mammalian species are very rare, the only other known example being *ZFY* (10). This result is consistent with pY53.3 reacting with functional sequences on the eutherian Y chromosome.

### Sequence Analysis of Y-Unique Sequences

The sequence of pY53.3 was determined by primer-walking. A search of the EMBL DNA sequence database failed to find any sequence related to pY53.3. But inspection of the pY53.3 (2.1 kb) sequence revealed two long open reading frames corresponding to 99 and 223



**Figure 10.3**

*a*, “Noah’s Ark” blot of *Hind*III-digested genomic DNA from male and female pairs of eutherian mammals, hybridized with the 0.9-kb *Hinc*II fragment of pY53.3. The probe detects male-specific fragments in human (2.1 kb), chimpanzee (~18 kb), rabbit (4.2 kb), pig (6.6 kb), horse (~10 kb), cattle (1.6 kb) and tiger (6.3 kb). Rabbit ♀ lane is slightly overloaded. *b*, Southern blot of: ♂, 46XY cell line, PGF (ref. 28); 4Y, 49XYYYY cell line, Oxen (ref. 30); ♀, 46XX cell line, WT49 (ref. 29); 4X, 48XXXX cell line, GM1416B (Coriell Institute for Medical Research, Camden, New Jersey, USA); H, hamster parent cell line, W3GH (ref. 31); X, hamster-human hybrid cell line containing the human X chromosome, CL2D (ref. 32); Y, hamster-human hybrid cell line containing the human Y chromosome, 853 (ref. 33). The filter was hybridized with the (0.9 kb) *Hinc*II fragment of pY53.3, which detects a 2.1-kb *Hind*III fragment in ♂, 4Y and Y, the intensity of the bands reflects the number of copies of the Y chromosome present. DNA size markers (kb), to the left.

**METHODS.** Genomic DNA (10 µg) was digested with *Hind*III, separated on a 0.8% agarose gel, transferred to Hybond N<sup>+</sup> (Amersham) and fixed in 0.4 M NaOH. Probe pY53.3 was labelled with <sup>32</sup>P and added to the filter in 5 × SSPE buffer, 5 × Denhardt’s solution, 0.5% SDS, 10% dextran sulphate, 200 µg ml<sup>-1</sup> denatured salmon-sperm DNA, and hybridized for 16 h at 65 °C. The filter was washed in 1 × SSC buffer, 0.2% SDS at 65 °C and autoradiographed for 2 days.

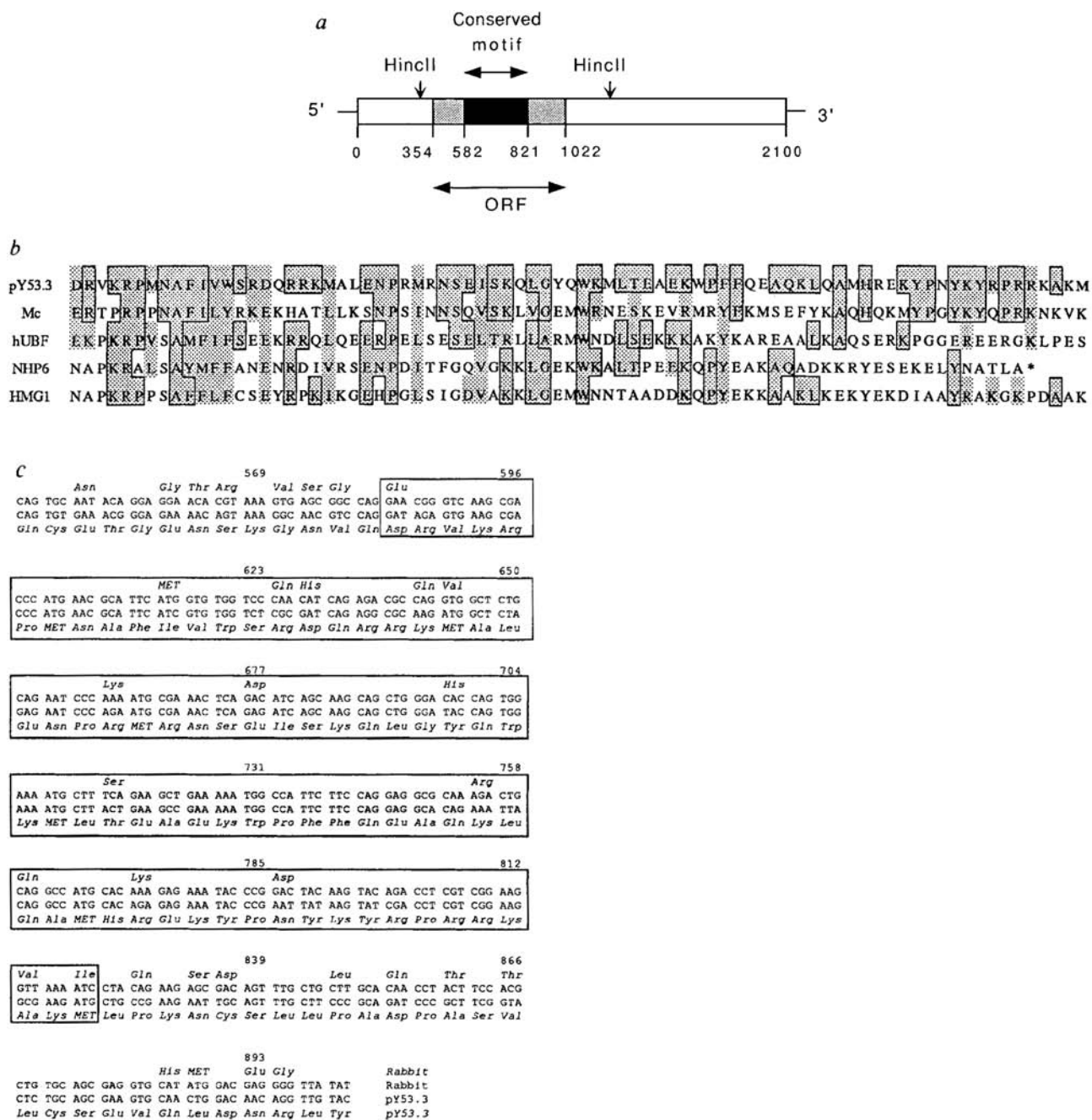


Figure 10.4

**a**, Diagram of pY53.3 (2.1 kb) subclone; shaded region, open reading frame (ORF). Black box, region covered by the 80-amino-acid conserved motif, which shows homology to the Mc protein of *S. pombe* and several non-histone proteins related to HMG1 and HMG2. Numbers represent base pairs and *HincII* sites define the 0.9-kb subclone used as a probe. **b**, Conserved 80 amino acids (single-letter code) of pY53.3 (human) compared with Mc protein from *S. pombe* (Mc), human upstream binding factor (hUBF), non-histone chromosomal protein (NHP6) from *S. cerevisiae* and high mobility group protein 1 (HMG1). Boxed regions, identical amino acids. Shaded regions, conservative amino-acid changes with respect to the human pY53.3 sequence. **c**, Nucleotide sequence of pY53.3 (human) and its corresponding amino-acid sequence compared with the rabbit Y-specific homologous sequences. The conserved motif of 80 amino acids is boxed.

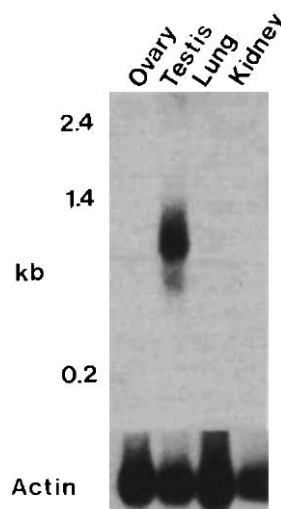
**METHODS.** Plasmids were subcloned in pUC18 vectors (NEB) and sequenced as double-stranded DNA by the dideoxy chain termination method (34) using synthetic oligonucleotide primers and Sequenase (USB).

amino acids that overlap in different frames, reading 5' → 3' from the centromere towards the pseudoautosomal boundary (figure 10.4a). We screened the PIR protein database using a similarity search algorithm for sequences related to the proteins predicted to be encoded by these open reading frames (18, 19). The protein putatively encoded by the shorter open reading frame was unrelated to any sequence in the PIR protein database. But the protein putatively encoded by the longer open reading frame was found to have striking similarity both to a portion of the Mc protein encoded by the *mat3-M* of the fission yeast *S. pombe* (20) and to a conserved motif in several non-histone proteins related to HMG1 and HMG2 (21, 22). The conserved motif covers a stretch of 80 amino acids and suggests a common structural role in these proteins, which could link them functionally (figure 10.4b).

At the 3' end of the conserved motif in pY53.3, the sequence continues for 68 amino acids before reaching a stop codon. No potential splice donor site was found in the DNA sequence of this region but there is a putative polyadenylation signal 133 bp downstream of the stop codon. In the 5' direction there is a potential splice acceptor signal in the pY53.3 DNA sequence at the point where homology between the conserved motif and pY53.3 ends. The open reading frame continues 5' for a distance corresponding to another 75 amino acids and within this region two potential start codons are found in pY53.3.

To test the conservation and hence functional importance of the sequence motifs in pY53.3, the homologous Y-specific rabbit sequence was cloned and sequenced. Within the conserved motif the human and rabbit Y-specific sequences share 64 out of 80 amino acids (80%) with a further eight amino acids showing conservative changes (90% similarity overall) (figure 10.4c). Outside the motif the human and rabbit show only 54% identity. The high degree of homology within the motif strongly suggests pY53.3 contains the coding sequence of a gene.

The other Y-unique probes found in the original search were also sequenced. The pNB and pY53.2 probes did not reveal any open reading frames predicted to encode proteins related to sequences in the EMBL database or the PIR protein database. The probe pY4.1β is part of a larger 1.2-kb *RsaI* fragment that contains an open reading frame predicted to encode a protein related to retroviral reverse transcriptase, commonly found in repetitive sequences. The probe pY53.1 encompasses a 5.6-kb region containing several open reading frames, but none of these was predicted to encode a protein related to sequences in the EMBL database or the PIR protein database. In total, 10.5 kb of the Y chromosome was sequenced in the search for potential coding sequence.



**Figure 10.5**

Northern blot analysis of poly(A)<sup>+</sup> RNA from ovary, adult testis, lung (male) and kidney (male), hybridized with the 0.9-kb *HincII* fragment of pY53.3. The probe detects a transcript of ~1.1 kb in adult testis and in no other tissue tested. Below is the same filter stripped and re-probed with β-actin, confirming the presence of poly(A)<sup>+</sup> RNA in all the tissue samples. Poly(A)<sup>+</sup> RNA was also prepared from three lymphoblastoid cell lines 49XYYY cell line (Oxen) (30), 46XY cell line (PGF) (28), 46XX cell line (WT49) (29), and probed as above, but no transcripts were detected (data not shown).

**METHODS.** RNA was prepared from each tissue as described previously (35) and poly(A)<sup>+</sup> messenger RNA selected by poly(A) tract isolation system (Promega). Poly(A)<sup>+</sup> RNA (8 μg) was separated on a 1% agarose gel containing 2.2 M formaldehyde, transferred to Hybond N (Amersham), ultraviolet-fixed, and hybridized with <sup>32</sup>P-labelled 0.9-kb *HincII* fragment of pY53.3. Hybridization was at 65 °C in 3 × SSC buffer, 5 × Denhardt's solution, 200 μg ml<sup>-1</sup> denatured salmon-sperm DNA, 6% polyethylene glycol and 0.1% SDS. The filter was washed in 1 × SSC buffer, 0.1% SDS at 65 °C and autoradiography was for 6 days at -70 °C. Conditions for re-probing the filter with β-actin were as above but the filter was washed in 0.1 × SSC buffer, 0.1% SDS at 65 °C and autoradiography was for 8 h.

### Tissue Distribution and Expression

A northern blot prepared with poly(A)<sup>+</sup> RNA from human tissues was hybridized with the 0.9-kb *HincII* fragment of probe pY53.3 (figure 10.5). The probe detects a fragment of about 1.1 kb in adult testis. Bands were not detected in ovary, lung (male) or kidney (male) cell lines, or in male and female lymphoblastoid cell lines. The filter was stripped and re-probed with β-actin to confirm the presence of poly(A)<sup>+</sup> RNA in the samples (figure 10.5). This result is consistent with a testis-specific transcript being encoded by the pY53.3 Y-specific sequence. This was confirmed using 3' RACE (rapid amplification of complementary DNA ends) polymerase chain reaction (PCR) (23) from adult testis poly(A)<sup>+</sup> RNA, which showed a poly(A) tract 15 bases downstream from the potential polyadenylation signal, further indicating that this is the 3' end of a Y-specific transcript (data not shown).

## Discussion

We have extended the findings of Palmer et al. (15) and defined a 35-kb region of Y-specific sequence immediately adjacent to the pseudoautosomal boundary in which *TDF* must reside. An extensive Southern blot analysis of this 35-kb region of the Y chromosome revealed a Y-unique probe, pY53.3, which detects conserved Y-specific sequences in a wide range of eutherian mammals. In related studies, Gubbay et al. (24) have demonstrated that the equivalent murine sequence is present in *Sxr'* (25)—the smallest part of the mouse Y chromosome known to be male sex-determining—and is deleted from a mutant Y chromosome that has lost male sex-determining function (26). The conservation of pY53.3-related sequences between the Y chromosomes of eutherian mammals suggests that these sequences have a functional role. The location of the pY53.3-related sequences on the Y chromosomes of man and mouse is consistent with a role in male sex determination.

The translated nucleotide sequence of pY53.3 (2.1 kb) contains two open reading frames. The longer open reading frame encodes a region of 120 amino acids shared with the homologous rabbit Y-specific sequence. Within this region there is a conserved motif of 80 amino acids which shows 80% identity, rising to 90% with conservative substitutions. Outside the conserved region the similarity between the residues drops to 54%.

The amino-acid sequence encoded by the longer open reading frame from pY53.3 showed striking similarity to 80 amino acids at the carboxy-terminal of the Mc protein encoded by the *mat3-M* locus of the fission yeast *S. pombe* (20). This is the same stretch of 80 amino acids in pY53.3 that is conserved in the rabbit sequence. The mating-type locus, *mat-1*, in the fission yeast has two alternative alleles, M and P. These alleles are transposed during switch of mating type from either donor loci *mat3-M* or *mat2-P* to the *mat-1* locus. Both loci contain two transposable genes (*Mc* and *Mi*, and *Pc* and *Pi*); none of the four genes are related to each other in sequence. The precise function of the four genes is not known; *Mc* and *Pc* are, however, required for mating and all four genes are needed for meiotic competence (20). By analogy to the budding yeast it has been suggested that genes of the *mat-1* locus function as transcription factors. This suggestion has been supported by the finding of a diverged homeobox domain in *Pi* (20).

The 80-residue conserved motif in pY53.3 also showed homology with a domain found in the nuclear non-histone proteins HMG1 and HMG2. High mobility group (HMG) proteins 1 and 2 are thought to play a part in chromosomal structure and gene activity, and some display enhanced DNA-binding to A + T-rich

single-stranded sequences (27). HMG1 and HMG2 are not known to regulate specific gene sequences but are associated with regions of transcriptionally active chromatin. Within HMG1 and HMG2 there is a motif, the HMG box, which has been found in the non-histone chromosomal protein NHP6 of *Saccharomyces cerevisiae* (22), the yeast ARS-binding protein, ABF2, and the human nucleolar transcription factor hUBF (human upstream binding factor) (21). The hUBF product is an RNA polymerase I transcription factor that interacts with sequence-specific DNA regions. This motif might represent a novel class of DNA-binding domains (21). The conserved binding motif seems to be present in a large family of related sequences perhaps originating from an early HMG-like nonspecific DNA-binding structure.

It is tempting to speculate that Mc is also a transcription factor and that the conserved motif shared with the human pY53.3 sequence is a DNA-binding domain. The only structural evidence to support this conjecture is the high Arg–Lys content of both Mc and the conserved motif of pY53.3, which is 25% Arg–Lys.

We have termed the human Y-located gene defined by pY53.3 *SRY* (sex-determining region Y). The gene has been defined with respect to its location because only the homology to other genes suggests a DNA-binding function. But the putative nucleic-acid-binding motif within *SRY* and its testis-specific expression are consistent with *SRY* having a role in the developmental regulation of the testis.

The presence of a 3' stop codon and a poly(A) tract in cDNA from 3' RACE PCR implies that the open reading frame in pY53.3 corresponds to the last exon of *SRY*. At the 5' end of the conserved motif sequence in *SRY* there is a potential splice acceptor site; but as homology between the rabbit and human genomic sequences continues past this site it may not represent an intron–exon boundary. This question will be resolved by the isolation and sequencing of transcripts from the *SRY* gene.

The 35 kb of Y-specific sequences immediately adjacent to the pseudoautosomal boundary are rich in repetitive sequences and this has hampered analysis. The open reading frame in pY53.3 is the only well-conserved sequence detected; but this approach could have failed to detect small exons, especially if they are close to repetitive elements or are only weakly conserved between species. Therefore, we cannot exclude the existence of genes other than *SRY* in the human sex-determining region.

We have described a novel, transcribed gene, *SRY*, present in the sex-determining region of man and mouse. Sequences homologous to *SRY* are located on the Y chromosome in all eutherian mammals tested.

*SRY* encodes a protein with a potential DNA-binding domain, which is shared with the Mc protein of the mating-type locus of *S. pombe* and several non-histone proteins related to HMG1 and HMG2. *SRY* is currently the best candidate for *TDF*, the male sex-determining gene in humans. Proof of identity between *SRY* and *TDF* will require mutational analysis of XY females or the production of sex-reversed transgenic mice.

### Acknowledgements

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Sexual dimorphism in mammalian embryogenesis provides a model of a developmental switch. The signal event in the male pathway, differentiation of an indifferent (or bipotential) gonad into a testis (1), is regulated in early embryogenesis by the Y chromosome (2). A regulatory gene has been mapped to the short arm of the Y chromosome (interval 1A1) by molecular analysis of human sex reversal (XX males and XY females) (3). This gene encodes SRY, a DNA binding protein that is conserved among mammalian Y chromosomes and is similar to members of the HMG box family of transcription factors (4). Whereas mouse Sry and some other HMG box proteins contain a recognized transcription activation domain, other mammalian SRY proteins (including human) do not (4). The mechanism of action of SRY is unknown.

The mouse homolog of SRY is selectively expressed in the common gonadal primordia of male gonads just before morphologic differentiation (3). Its transgenic expression in XX mice is sufficient to induce a cascade of downstream regulatory events that lead to formation of male-specific structures and regression of female primordia (5). Although the steps of this pathway and their regulatory relationships are incompletely delineated, testicular morphogenesis is characterized by secretion of MIS by Sertoli cells (6) and of testosterone by Leydig cells (1). MIS induces regression of the Müllerian duct (the anlagen of the uterus, fallopian tube, and upper vagina); androgenic steroids induce masculinization of the external genitalia and differentiation of the Wolffian duct (the anlagen of the vas deferens, seminal vesicles, and epididymis).

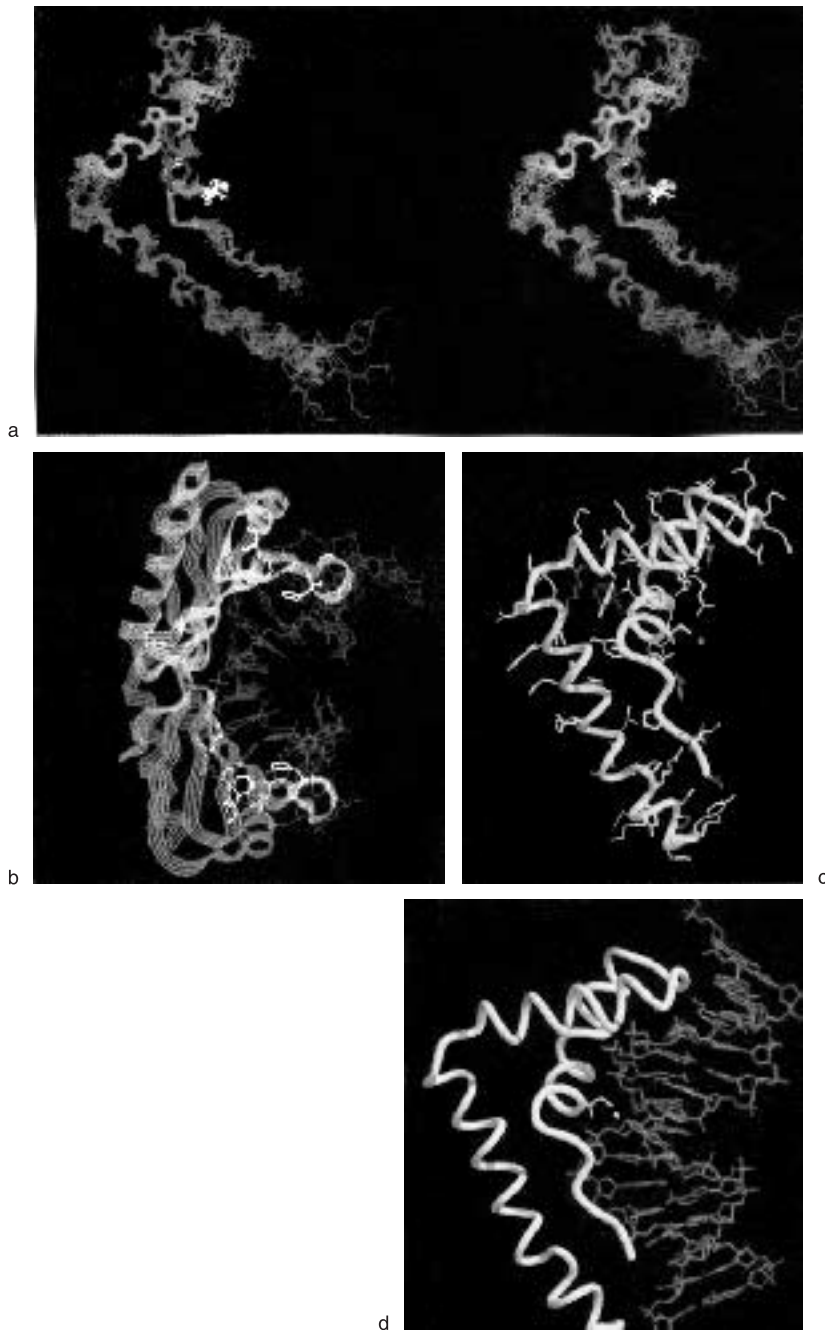
Progress in reproductive biology has traditionally been stimulated by interdisciplinary analysis of patients with altered or ambiguous sexual development. Together, observations of clinicians, geneticists, and biochemists have demonstrated that expression of the complete male phenotype requires a complex series of steps. Examples of clinical disorders and their underlying mechanisms include complete sex reversal caused by mutation or translocation of the SRY gene (7), retained Müllerian duct syndrome resulting from mutations in MIS (8), and male pseudohermaphrodi-

tism associated with defects in the androgen receptor (9) or testosterone 5 $\alpha$ -reductase (10).

We and others have used mutations in SRY in patients with 46,XY pure gonadal dysgenesis (7) to investigate initial molecular events in testicular differentiation. The components identified in this developmental pathway can define the mechanisms underlying the molecular events that lead to sexual differentiation. Correlated studies of structure and function illustrate how such mutations affect this decision at the levels of protein folding, DNA recognition, and transcriptional regulation. In complementary studies of human or rat *MIS* promoters transfected with human *SRY* into an immortalized urogenital ridge cell line we investigated how SRY activates a male-specific transcription pathway. These investigations define a mechanism by which SRY recognizes DNA, demonstrate an SRY-dependent pathway of *MIS* gene expression, and provide evidence for an intervening factor or factors (designated SRYIF) interposed between SRY and activation of the *MIS* promoter.

#### The HMG Box: DNA Recognition by Side Chain Intercalation

SRY contains a conserved DNA-binding domain, the HMG box (11). Binding occurs primarily in the DNA minor groove (12) and induces a sharp bend (13). The structure of an HMG box contains three  $\alpha$  helices with L-shaped orientation (14, 15); its angular protein surface presumably provides a template for DNA bending. Because the structure of SRY has not been determined, we have constructed a model of its DNA-binding domain on the basis of nuclear magnetic resonance (NMR) solution structure of rat HMG domain 1B (14, 16). With conservative assumptions, distance-geometry and simulated annealing (DGSA) (17) yielded a single family of structures (figure 11.1A) that contained both similar secondary structure and hydrophobic core (18, 19). The models retained an angular tertiary structure, defining convex and concave surfaces (14). Mutations in SRY associated with human sex reversal (7) are clustered in the HMG box; these mutations (figure 11.1B) are predicted to occur both in



**Figure 11.1**

(A) Homology models of the human SRY HMG box (stereo pair); an ensemble of ten structures is shown. I68 and F67 are shown in yellow and red, respectively [SRY residue numbers refer to the full-length sequence of the human protein (7)]. (B) Wire model with side chains: I68 (yellow); other sites of intersex-associated mutations (red), SRY residues V60, R62, M64, I68, W70, M78, P83, I90, K92, G95 (not shown), K99, L101, K106, and P125 (7, 51a) and unaffected sites (white). (C) Co-crystal structure of TBP-DNA complex: protein (green) and bent DNA (red). Two pairs of phenylalanine side chains insert between base pairs at kinks [(yellow; coordinates) adapted from (22)]. (D) Schematic alignment of the concave surface of the HMG box and bent DNA. The intercalating isoleucine (yellow) is placed at a kink in the DNA (red arrow) (52).

the hydrophobic core and on the protein surface. Several of these mutations decrease specific DNA binding (7).

The inferred structure of the HMG box of SRY offers two possible surfaces for DNA recognition—convex (Model I) or concave (Model II) (figure 11.2A). The models predict different associations between the DNA and protein. In Model I the DNA would bend toward the protein [as observed with the catabolite activator protein (CAP) from *Escherichia coli* (20) and the eukaryotic nucleosome core particle (21)]. In Model II the DNA would bend away from the protein [as observed with the TATA-binding protein (figure 11.1C) (22, 23)]. To distinguish between these models, we used a clinical mutation (I68T) (7) to identify a direct contact between the HMG box of SRY and the bent DNA site. This mutation caused sex reversal in a female patient with a 46XY karyotype. The DNA used was a variant of a high-affinity SRY-binding site in the human *MIS* promoter (24–26).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
5'	G	G	G	G	T	G	A	T	<b>T</b>	<b>G</b>	T	T	C	A	G
3'	C	C	C	C	A	C	T	A	<b>A</b>	<b>C</b>	A	A	G	T	C

NMR studies comparing complexes of wild-type and mutant proteins with DNA (27) revealed that an isoleucine side chain contacts the DNA minor groove and inserts between specific AT base pairs (figure 11.2C). The distal methyl resonance of the isoleucine ( $\delta$ -CH<sub>3</sub> in figure 11.2D) is shifted to high field, presumably by DNA ring currents, and exhibits nuclear Overhauser effect (NOE) contacts with base protons in the DNA interior (N<sup>3</sup> imino protons of T8 and T9, circled in the central panel of figure 11.2B). Insertion of an isoleucine side chain by wild-type SRY disrupts base stacking (as indicated by attenuation of NOEs and loss of neighboring ring-current effects); base pairing is maintained (27). This interaction would be expected to alter torsion angles in the DNA backbone and the global direction of the double-helical axis.

To identify the inserted side chain and thus the DNA-binding surface, substitutions were introduced into each of the possible DNA-binding isoleucines [I68T and I90V in full-length human SRY (7)]. The mutant domains were properly folded, and their thermodynamic stabilities were similar to that of the wild-type protein (figure 11.2E). The NMR spectrum of the I90V complex retained an isoleucine spin system shifted to high field whereas this spin system was absent in the spectrum of the I68T complex (figure 11.2D). When the model was compared to similar NMR structures (14, 15), it was apparent that residue 68 projects from  $\alpha$  helix 1 to define the inner crux of the convex surface (figures 11.1 and 11.2A); by con-

trast, residue 90 packs into the hydrophobic core. Assignment of I68 as the inserted side chain excludes Model I and supports Model II. A schematic model of an SRY-DNA complex (figure 11.1D), juxtaposes the concave surface of the HMG box with a bent DNA site. Docking of helix 1 and insertion of I68 is expected to widen the minor groove relative to that of B-DNA.

The inserted isoleucine is critical for DNA recognition. Specific binding to the ATTGTT site was tested in a gel-retardation assay (figure 11.2E). Whereas the I90V substitution did not affect specific DNA binding, the binding affinity of the I68T mutant was less than one-fiftieth that of the wild-type protein (28). The assay detected complexes with a broad range of electrophoretic mobilities (figure 11.2E, part a, lane 5), which presumably represent dissociating I68T protein-DNA complexes. Kinetic instability of the variant complex was directly demonstrated by <sup>1</sup>H-NMR spectroscopy. On addition of the I68T protein, DNA resonances exhibited fast exchange between free and bound chemical shifts, indicating an exchange lifetime of <5 ms at 25°C; in contrast, the wild-type complex exchanged slowly (lifetime > 200 ms). We imagine that the inserted isoleucine serves to lock the DNA into a specific structure. Because the I68T substitution is associated with human sex reversal (46,XY pure gonadal dysgenesis), a correlation is obtained between a molecular mechanism and a developmental phenotype. However, position 90 can also be a site of clinical mutation [I90M (7)], presumably as a result of altered protein folding or stability (29).

Systematic variation of oligonucleotides around a protected site TTTGTG (24) of SRY-DNA interaction (figure 11.3A) led to identification of an optimal ATTGTT site (26, 27). Indeed, almost all single base changes from the ATTGTT site resulted in decreased binding (figure 11.3B). Specificity was most stringent in the central TT (figure 11.2C), the site of penetration by the nonpolar I68 side chain (30). We also performed complementary studies of phosphate contacts (figure 11.3C). Site-specific interference with SRY binding was observed after incorporation of methylphosphonate, a neutral and nearly isosteric analog of the phosphodiester linkage (31). The results demonstrated that each phosphate in the ATTGTT site contributes to the protein-DNA interaction. This pattern—contiguous phosphate contacts across both strands—is different than that observed with major groove DNA-binding motifs (32). To align the extensive SRY-phosphate contacts along a single face would require partial unwinding of the double helix. The inferred DNA distortions (bending, partial unwinding, and widening of the minor groove) predict marked dispersion of <sup>31</sup>P-NMR resonances (33). This was indeed observed in the SRY-DNA complex (figure 11.3D).

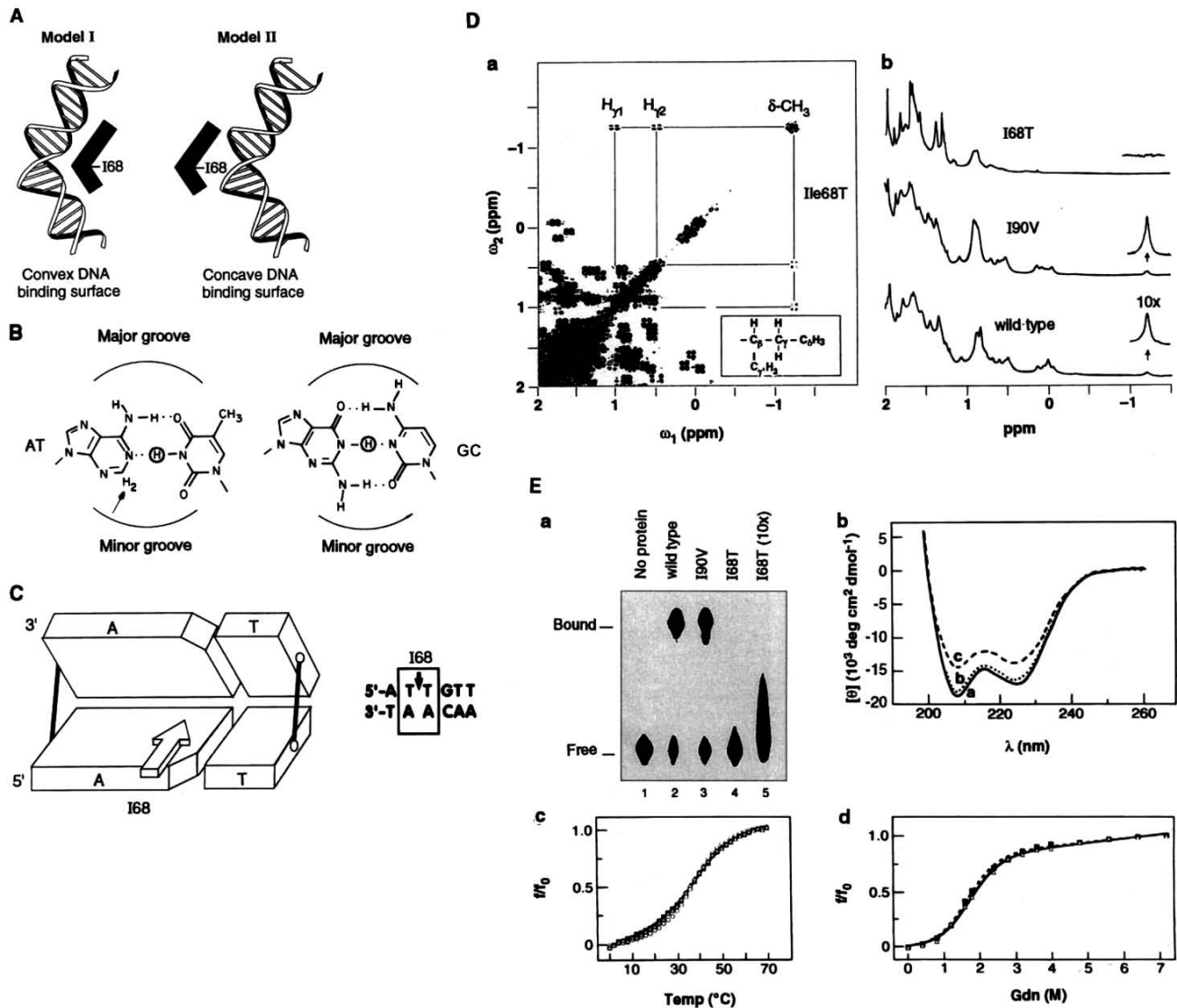
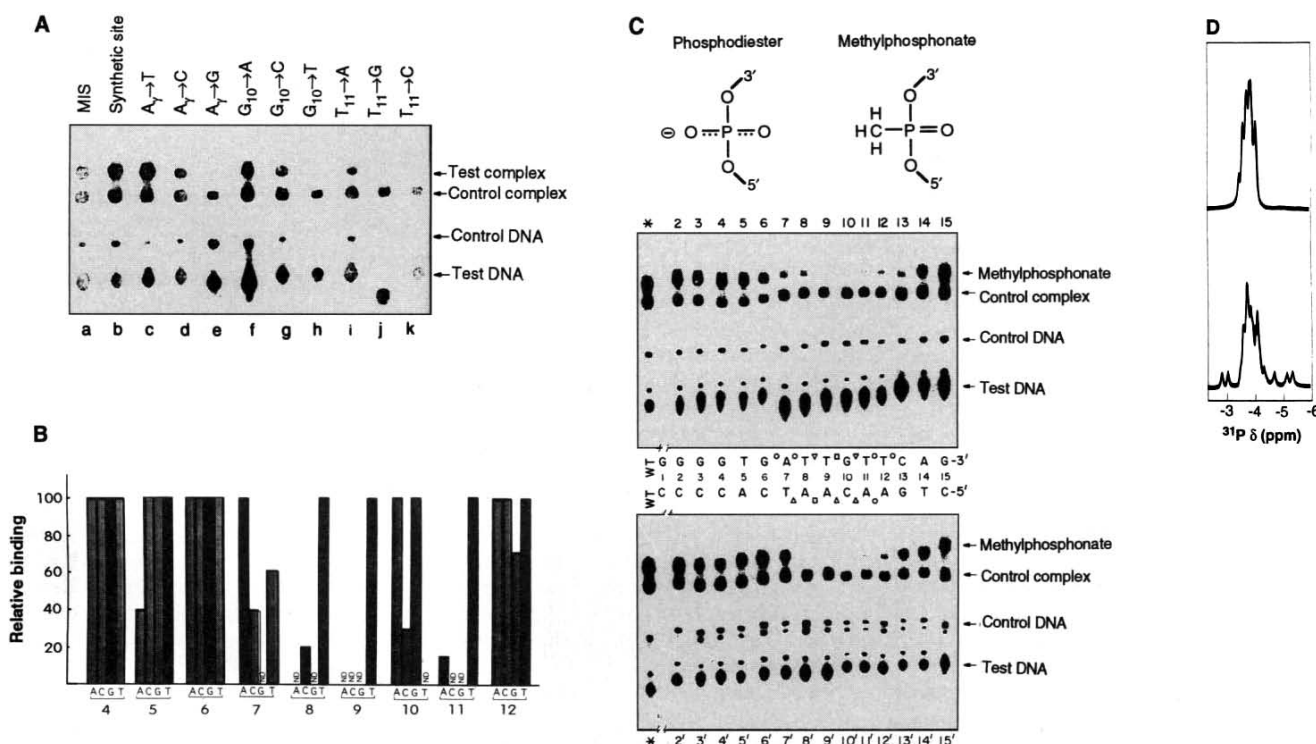


Figure 11.2

(A) Possible mechanisms of binding between the SRY HMG box and DNA. Model I, convex protein surface; Model II, concave protein surface. (B) Chemical structure of AT and GC base pairs; imino protons are circled. AT and GC base pairs are distinguished in the minor groove by presence of adenine H2 protons (arrow) or bulkier guanine 2-NH<sub>2</sub> group. Close contact between I68 and H2 of A<sup>9</sup> precludes guanine at this position. (C) Model of I68 insertion. Insertion disrupts base stacking but not base pairing. The target sequence and insertion site are shown at right; the two base pairs flanking the insertion site are boxed. Partial intercalation requires a widened minor groove (27). (D) (a) 500 MHz DQF-COSY  $^1\text{H}$ -NMR spectrum of specific 1:1 complex between SRY HMG box and duplex DNA site 5'-GGGGTGATTGTTTCAG-3'. The upfield  $\delta$ -methyl and  $\gamma$ -methylene spin subsystem of I68 is outlined. The pattern of the through-bonded  $^1\text{H}$ - $^1\text{H}$  correlation (solid line) is unique to the isoleucine side chain (53). (Inset) structure of the side chain with protons labeled. (b) Assignment of inserted isoleucine to SRY position 68 by site-directed mutagenesis (54–56). Aliphatic regions of 1D  $^1\text{H}$ -NMR spectra of native (bottom), I90V (middle), and I68T (top) complexes; the amplitude (y-axis) of the upfield I68  $\delta$ -CH<sub>3</sub> resonance is enlarged tenfold as indicated in lower two spectra (arrows). (E) (a) Gel mobility-shift assay. Free and bound bands are as indicated at left. Lane 1, no protein; lane 2, 25 nM wild-type SRY-p2; lane 3, 25 nM I90V variant; lane 4, 25 nM I68T variant; lane 5, 250 nM I68T variant (57). (b) CD spectra at 25°C of wild-type and mutant SRY domains: wild type [solid line (a)], I68T [dotted line (b)], and I90V [dashed line (c)]. The I68T substitution appears to be structurally conservative whereas I90V is associated with an apparent 25% decrease in  $\alpha$  helix content (58). (c) Thermal denaturation of wild type ( $\square$ ), I68T ( $\circ$ ), and I90V ( $\triangle$ ) as monitored by CD at 222 nm (59, 60). (d) Guanidine-HCl denaturation studies of wild type ( $\triangle$ ; solid line) and I68T ( $\square$ ; dashed line) SRY domains as monitored by tryptophan fluorescence (61). Temp, temperature.

**Figure 11.3**

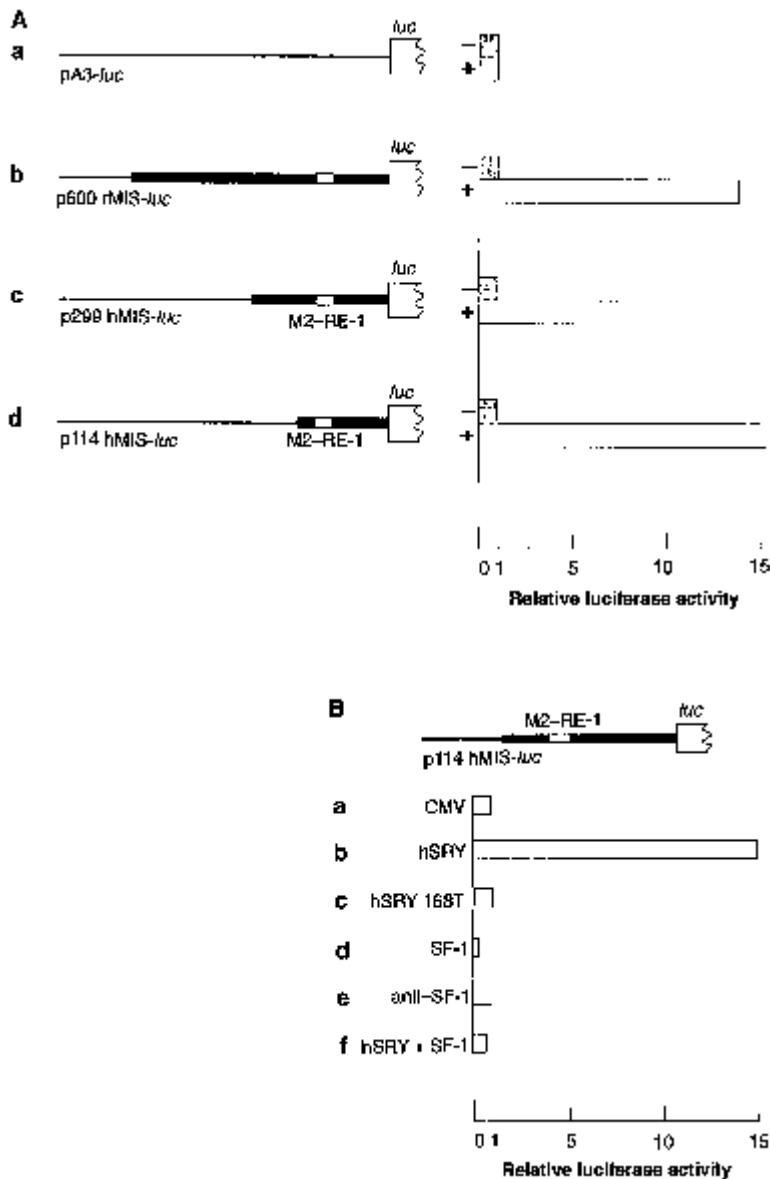
(A) Representative gel showing SRY-HMG box binding to a duplex 15-bp DNA sites. In each lane SRY-p2 binding to a variant 15-mer DNA site (test site) can be compared to binding to the 36-mer parent site (an internal control). The optimal site to be analyzed (lane B) is 5'-GGGGTGATTGTTTCAG-3', which was also used in NMR studies (figure 11.2D). The control DNA is a 36-mer duplex (5'-CATACTGCGGGGGTGATTGTTTCAGGATCATACTGCG-3'). Shown in lane a is SRY-p2 binding to the MIS promoter sequence 5'-GGGGTGTTTGTGCAG (25), whose affinity for SRY is less than one-eighth that of the optimal SRY-binding sequence (62). (B) Relative binding strengths of variant DNA sequences. Binding to the optimal 5'-GGGGTGATTGTTTCAG-3' sequence is normalized to 100. ND, no specific binding detectable. (C) Gel retardation assay of methylphosphonate-modified 15-mer DNA duplexes. Experimental conditions were the same as in (A). Chemical structures of phosphodiester and methylphosphonate backbones are outlined (upper panel). (D) <sup>31</sup>P-NMR spectra of duplex 15-mer DNA (upper tracing) and 1:1 SRY-p-15 mer DNA complex (lower tracing). <sup>31</sup>P chemical-shift perturbations (near -3 and -5 part per million) indicate significant distortion of torsion angles in DNA backbone at least at five sites (63).

An analogy can be drawn between the HMG box of SRY and the eukaryotic TATA-binding protein (TBP) (figure 11.1C) (22, 23). Although they exhibit distinct structural motifs, the two proteins share (i) use of a concave surface to bind and bend specific DNA sequences, (ii) docking of protein-secondary structure in a widened and underwound minor groove (figure 11.1C and D), (iii) extensive protein-phosphate contacts with both DNA strands, and (iv) penetration of nonpolar or aromatic side chains between specific base pairs, disrupting base stacking but not base pairing. Moreover, TBP, like SRY, binds not only to a consensus site but also to a family of variant sequences. Other components of the basal transcriptional machinery work cooperatively with TBP to extend its specificity (34-36).

#### From Structure to Function: Activation of the MIS Pathway by SRY

To define the functional properties of SRY in regulating transcription, expression and reporter plasmids

were transfected into a cell line, CH34, derived from the differentiating gonadal ridge of male rat embryos (37). Human SRY, unlike mouse Sry, has no transcriptional activation domain, suggesting that cofactors may be necessary to mediate its action at target promoters (4). Because the CH34 cell line is derived from tissue at the specific site and stage of SRY expression in embryogenesis, it is likely to contain the tissue-specific accessory machinery necessary to respond to SRY. Expression of MIS (38) just after the period of SRY expression in the indifferent gonad (1, 39) is an early landmark in male-specific gene transcription. The MIS promoter can thus provide an assay for SRY-dependent activation of a pathway of male gene expression. To define the regulatory sequences required for enhanced transcription at the MIS promoter, a series of deletions of the human MIS promoter were constructed and tested for response to SRY (figure 11.4A). Fifteenfold activation was conferred by the proximal 114 base pairs (bp) of the human MIS promoter (figure 11.4A) when transfected with SRY into CH34 cells. No activation of the control reporter vector was observed



**Figure 11.4**

Response of MIS promoter to full-length human SRY in CH34 immortalized urogenital ridge cells (64): (A) The indicated reporter constructs were transfected together with pCMV (–) or pCMV-hSRY (+) (65): (a) promoterless pA3-luc, (b) p600rMIS-luc containing a 600-bp rat MIS promoter, (c) p299hMIS-luc containing a 299-bp human MIS promoter, or (d) p114hMIS-luc containing a 114-bp human MIS promoter responsive to SRY (71). Transcription activity is displayed relative to the amount obtained with empty expression vector pCMV (–) and empty reporter vector pA3-luc (1X). (B) Response of the MIS promoter (top) to wild-type SRY, mutant SRY derived from a sex-reversed human patient, and SF-1 in urogenital ridge cells. (a) Cells were transfected with reporter vector p114hMIS-luc along with pCMV (a), wild-type SRY (b), mutant SRY I68T (c), SF-1 (d), antisense SF-1 (e), or (f) both SRY and SF-1 (72).

(figure 11.4A). In addition, no regulation of a Rous sarcoma virus promoter by SRY occurred (39a). Thus, SRY can specifically activate a physiologically relevant target gene in a tissue-culture model of the differentiating gonad.

Mutation I68T in the SRY DNA-binding domain, which changes the length and polarity of the inserted side chain, also reduced the transcriptional response when transfected with a wild-type MIS-reporter vector (figure 11.4B). Thus, this mutation, which is seen in a patient with 46XY clinical sex reversal and is critical for the mechanism of SRY recognition of the 5'-ATTGTT consensus site, also abolishes transcriptional activation of a male-specific pathway in an embryonic cell line. This correlation supports the hypothesis that the genetic role of SRY in testicular differentiation requires specific DNA binding by the proposed mechanism of partial side chain intercalation.

Although the 114-bp human MIS promoter contains a single SRY protected region (−45 to −75) (24), mutation in this region did not diminish the transcriptional response (39a–41), but did abrogate binding of SRY to the promoter. These observations indicate that, although SRY can function as a transcription factor, SRY-dependent activation of the MIS promoter in an embryonic gonadal cell line is indirect. Therefore, we postulate the existence of an intervening SRY-induced factor or factors, designated SRYIFs, that transduce the SRY signal to the responding MIS promoter.

The 114-bp MIS promoter contains regulatory regions conserved in the promoter of MIS from distantly related species. One such region (M2), that contains the steroidogenic factor-1 (SF-1) MIS-RE-1 site, can bind tissue-specific and developmentally regulated factors (42). SF-1 is a candidate SRYIF on the basis of observations that (i) SF-1 activates MIS transcription in 20-day primary postnatal Sertoli cells (43) and (ii) transgenic male homozygously deleted of SF-1 lack gonadal development (44). In the CH34 cell line, however, SF-1 failed either alone or in combination with SRY to elicit expression of the MIS reporter (figure 11.4B). In fact, SF-1 decreased basal expression from the MIS promoter to 35% of that of the antisense or empty vector controls (figure 11.4B). This inhibition and the ability of SF-1 to block MIS induction by SRY indicates that SF-1 may be a transcriptional repressor in this context.

Further evidence that SF-1 is a transcriptional repressor in the urogenital ridge cell line came from mutagenesis of the MIS-RE-1 site. Mutation of the TC at positions −95 and −96 to CT caused a decrease in binding of SF-1 to the MIS target site in gel shift experiments (43). With a 114-bp MIS reporter containing this mutation (40), however, SRY enhanced tran-

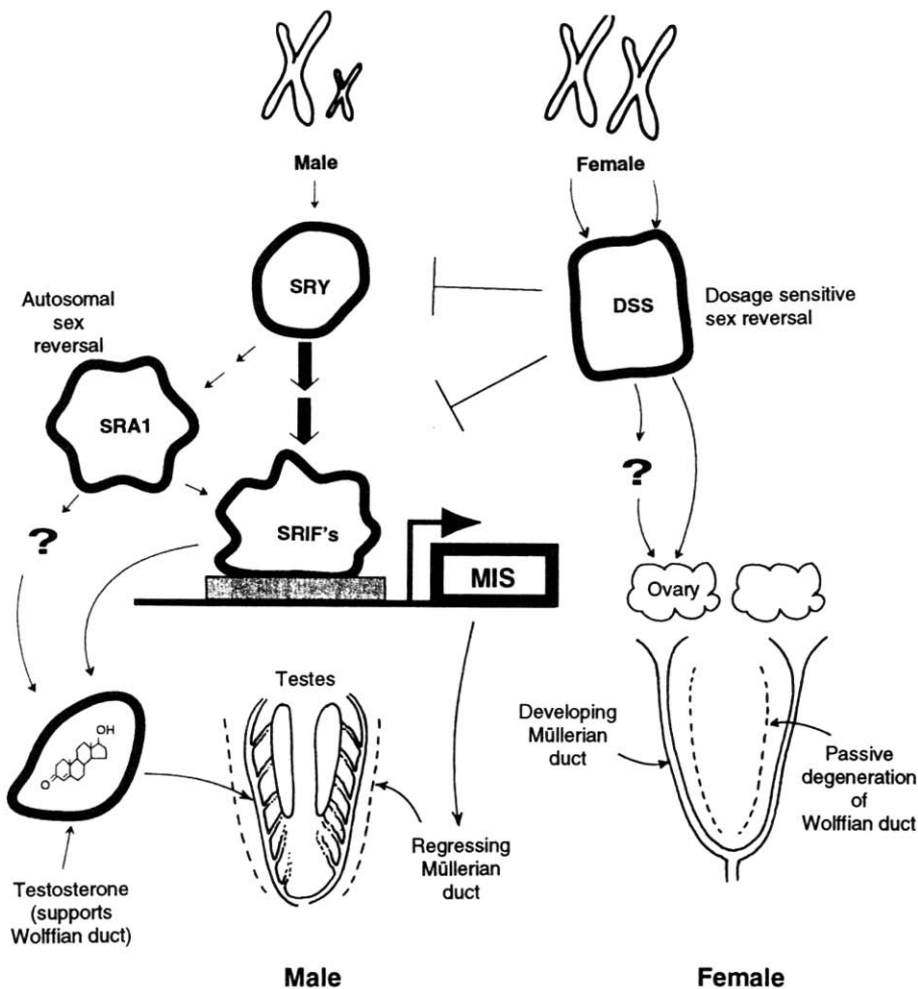
scription 39-fold rather than the 15-fold, as seen with the wild-type promoter.

SRY activates a pathway that leads indirectly to expression of the MIS gene promoter. Such activation occurs in an appropriate developmental context, a male urogenital ridge cell line, through a small region of the proximal MIS promoter. Mutational analysis of that region should further delineate the mechanism of SRY action.

### Summary and Perspectives

Molecular and cellular model systems have been used to address mechanisms by which SRY recognizes specific sites in DNA and activates downstream gene expression, and to examine how structure and function can be correlated in testis determination. The information currently available supports the following molecular principles: (i) SRY binds and bends DNA by means of the concave surface of an  $\alpha$ -helical structure (figure 11.2A, Model II). Sequence specificity requires insertion of a nonpolar side chain through the DNA minor groove, disrupting base stacking but not base pairing. In human SRY, mutation of this isoleucine is associated with clinical sex reversal. (ii) Expression of SRY in an embryonic testicular cell line initiates transcription of MIS indirectly through the 114-bp promoter. (iii) Mutation of full-length SRY at position 68, the site of partial side chain intercalation, abolishes its ability to induce transcription of MIS. (iv) Analysis of regulation through the proximal MIS promoter indicates that intervening transcription factors exist that may directly interact with the 114-bp region of the MIS promoter (figure 11.5) or influence the basal transcription machinery.

Sexual differentiation in mammals requires a precise choreography of molecular and cellular events. The initial step—establishment of gonadal sex in the previously undifferentiated urogenital ridge—provides a general model of a genetic switch in organogenesis. Identification of SRY as the testis determining factor (3) permits biochemical characterization of this switch. Its expression is appropriately specific for sex (male), tissue (urogenital ridge), and stage of development (just prior to morphologic differentiation of the testis) (3). Although its biochemical mechanism is not well understood, SRY belongs to a family of HMG transcription factors and can itself activate transcription (4, 45). Selective binding of SRY to responsive promoters by the mechanisms we have observed may be further enhanced by local changes in DNA structure (as influenced by superhelical density and nucleosome phasing) and by cooperative binding of other transcription factors (46, 47). Sharp DNA bends induced by SRY binding are proposed to organize the higher order



**Figure 11.5**

A hypothetical scheme for sex determination in mammals. The male regulatory pathway is initiated by the sex regulator on the Y chromosome, SRY, which acts to influence gonadal morphogenesis leading from an indifferent pattern of support cells surrounding germ cells to a male pattern of dispersed support cells surrounding germ cells in seminiferous tubules. A factor or factors (SRYIFs) mediates induction of the MIS gene by SRY. Although we have illustrated SRYIF interacting with the MIS promoter, an indirect interaction is also possible, for example, through modification of the basal transcription machinery. MIS, in turn, plays a key role in male sexual development as a diffusible substance causing regression of the female Müllerian duct derivatives—the uterus, fallopian tubes, and vagina.

structure of promoters (12, 48), thereby modulating assembly of more complex protein-protein interactions.

Molecular and cellular model systems can provide insight into mechanisms of gene regulation, but do not capture the complexities of organogenesis. How do such molecular pathways, once defined, orchestrate cell-cell interactions in testicular differentiation? The mature testis is a dynamic matrix of Sertoli, Leydig, myeloid, and germ cells that provides an environment for elaboration of growth and differentiating factors and for spermatogenesis in adult life. Future dissection of testicular morphogenesis will require identification of those genes intermediary between SRY and MIS in the male pathway (figure 11.5). SRY is proposed to directly activate target genes that may include, for example, the autosomal sex reversal factor SRA-1 [linked to

campomelic dysplasia on chromosome 17q in humans (49)], or the canine autosomal sex reversal gene (50). Complementary analysis of sex-reversed females with intact SRY has led to identification of a locus on the short arm of the X chromosome at which duplication is associated with female differentiation. This locus, designated DSS (dosage-sensitive sex reversal), may act either by repressing the SRY-induced male pathway or by activating expression of genes required for formation of female structures (51). SRYIF may be one of several factors participating with SRY in the determination of gonadal sex, as suggested by the phenomenon of sex reversal associated with abnormalities on mouse chromosome 17 T (51). Combined application of mammalian genetics, biochemistry, and molecular biology promises to define more fully the initial, inter-

mediary, and distal downstream steps of the male and female developmental programs. Such information may have broad clinical implications for infertility, contraception, and control of gonadal tumors.

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40. Mutant plasmids were constructed as described (figure 11.4A) with new PCR primers. For mutation of nucleotides -95 and -96, primers CMH-141 5'-GGTACCGGGCACTGTCCCCAAGGC-TGCGGCA GAGG-3' and CMH-107 were used. To construct the SRYe mutation, the promoter was rebuilt from two PCR products ligated at the Apa I restriction site. The 5' fragment used primers CMH-128 with CMH-119 5'-GATGGGCCCCAGGACAGACC-CCTATCTCCTCTGC-3' and the 3' fragment was made from annealing oligomers CMH-111 5'-CGCGGGCCCCACCCACCT-TCCACTCGGCTCACTTAAGGCAGGCAGCCAGCCCTGG-CAGCACCCAAGCTTCCG-3' and CMH-112 5'-CGGAAGCTT-GGGTGCTGCCAGGGGCTGGGCTGCCTGCCTTAAGTGAG-CCGAGTGGAAGGTGGGGTGGGGCCCGCG-3'. The resulting promoter was subcloned into the Hind III site of pA3-luciferase (41).
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52. Figures were generated with the Quanta (Molecular Simulations, Inc.) and Mitos (UCSF Graphics Laboratory) software packages. The DG-SA homology model was built with the DGII program (T. F. Havel, Harvard Medical School). We used 22,005 distance and 226 dihedral restraints as follows: (i)  $d_{NN}$ , ( $i, i+3$ ), ( $i, i+4$ ), and ( $\phi, \psi$ ) restraints (total 349) imposed corresponding  $\alpha$ -helical segments. Distances were constrained to within  $\pm 10\%$  of their ideal values; dihedral angles were constrained to within  $\pm 20^\circ$ . (ii) Interatomic distances between identical side chains (19 positions; 25%) were constrained within the range seen in the NMR ensemble of HMG1 (14) as extended by  $\pm 0.15$  Å (total 21,698). (iii)  $\chi^1$  and  $\chi^2$  dihedral angles of buried identical side chains were constrained to their ranges in the NMR ensemble of HMG1  $\pm 20^\circ$ . (iv) Conservative substitution (eight sites; for example, L69V and Y109F) received analogous  $\chi^1$  dihedral restraints  $\pm 20^\circ$ . For aromatic rings, analogous  $\chi^2$  dihedral restraints were imposed for identical residues. There were no restraint violations in the final ensemble.
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54. The SRY HMG box (designated SRY-p; 85 residues) used in panel a was expressed in *E. coli* strain BL21 and purified as described (27). The 1:1 peptide-DNA complex was made 2 mM in 10 mM potassium phosphate (pH 6.0). Site-directed mutations were introduced into an M13 construct by the Kunkel method (55) with the primers: I68T, CMH-081a 5'-GCGAGACCACACGGCGAATGCGTTC-3'; and I90V, CMH-079 5'-GCTGCTTGCTCACCTCTGAGTTTCG-3'. To increase efficiency of expression and to facilitate purification, wild-type and mutant HMG domains (panel b) were recloned by PCR into pTSN53 [J. Markley (56)]. A thrombin cleavage site and a His<sub>6</sub> affinity tag were introduced into respective 5' and 3' ends of the SRY coding region by PCR primer design. Final constructions were verified by double-stranded DNA sequencing. The His<sub>6</sub> fusion proteins (designated SRY-p2) were purified by affinity chromatography (Ni-NTA column; Qiagen, Inc.). The SRY HMG box was removed from the staphylococcal nuclease by thrombin digestion and isolated by SP-Fast Flow cation exchange chromatography (Pharmacia, Inc., La Jolla, CA). The DNA binding properties of the SRY HMG box are identical in the absence or presence of the COOH-terminal His<sub>6</sub> tag. The 15-base oligonucleotides were purchased for NMR study from Pharmacia, Inc., (Milwaukee, WI); purity was >98% as assessed by gel electrophoresis.
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57. <sup>32</sup>P-labeled duplex DNA and SRY-p were incubated at 20°C in 10 mM potassium phosphate (pH 7.4), 50 mM KCl, 2 mM MgCl<sub>2</sub>, 10% glycerol for more than 2 hours. Complexes were resolved on a polyacrylamide gel (6%). A 15-mer DNA site 5'-GGGGTGATTGTTTCAG was used.
58. CD spectra were obtained at 25°C with an Aviv spectropolarimeter; the protein was made 25  $\mu$ M in 200 mM NaCl, 10 mM sodium phosphate (pH 6.0). A 0.1-cm path-length cell was used and incubated for approximately 5 min before data acquisition. Final spectra were obtained by averaging 6 multiple-scan spectra acquired with a scan speed of 10 s at each wavelength.
59. For thermal unfolding studies, samples in 0.1-cm CD cell were equilibrated for 10 min at 0°C followed by 2-min equilibration, and 60-s data acquisition. The procedure was repeated successively at 2° temperature increments. Theoretical curve (solid line) was fit by the least squares procedure to the equation,  $\Delta G = \Delta H - (T/T_m)(\Delta H_1 - \Delta G_1) + \Delta C_p[T - T_m - T \times \ln(T/T_m)]$  (60). In each case  $\Delta H_1 = 27 \pm 3$  kcal/mol and the  $T_m$  is approximately 39°C.
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61. Excitation wavelength 295 nm (slit width 5 nm) and emission wavelength 350 nm (slit width 10 nm) were used. Wild-type and mutant proteins were made 1  $\mu$ M in 50 mM NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 6) in various concentrations of guanidine HCl. Experimental curves were fitted to the equations:  $\Delta G = \Delta G_U + m \times [\text{Gdn}] = -RT \times \ln K_e$ . An extinction coefficient of  $E_{280 \text{ nm}} = 2.11 \times 10^4$  mol<sup>-1</sup> cm<sup>-1</sup> was used for both wild-type and mutant samples. In the two-state model (60)  $\Delta G_U$  for both wild type and I68T are  $\approx 2.3$  kcal/mol.
62. Gel mobility-shift assays was performed as described in (57).
63. Both samples were made 0.625 mM in D<sub>2</sub>O with 2.33 mM potassium phosphate (pH 6.4) and 11.66 mM potassium chloride. The spectra were taken at 40°C with a 10-mm probe.
64. Urogenital ridge cell line CH34 was derived from the urogenital ridge of 14-day-old embryonic rats euthanized under institutional protocol MGH #A3596-01. Forty ridges were minced with a razor blade, triturated in 0.1% trypsin for 30 min at 37°C, and allowed to attach to tissue culture plastic for 4 hours. Infections with  $\psi$ 2 retrovirus harboring the v-myc oncogene and the neomycin antibiotic resistance gene (S. Fields-Berry and C. Cepko) were done with standard techniques (70). Immortalized G418-resistant colonies were analyzed for expression of SRY, MIS, and MIS receptor by RT-PCR and Northern (RNA) analysis. Clone CH34 expressed both SRY and the MIS type II receptor.
65. To create pCMV-hSRY, full-length 204-amino acid human SRY was isolated from pDPI327 (D. Page, Whitehead Institute) with oligos CMH-066 5'-ACCGGATCCATGCAATCATATGCTTCTGC-3' and CMH-067 5'-GGCGGATCCGGTACCGATTGTCTACAGCTTTG-3' for PCR, cloned into TA vector (Invitrogen Inc), sequenced and subcloned into the Bam HI site of pCMV (66). The 3' sequence of pCMV-hSRY was designed to alter T202P. All calcium phosphate transfections were done as described (67) with 1- $\mu$ g expression vector, 2- $\mu$ g reporter vector, and 0.5- $\mu$ g pXGH5 (68), an expression vector directing human growth hormone expression for use as an internal standard. Average Luciferase activity of three independent transfections was determined, after freeze-thaw lysis (69), with Luciferin substrate according to the manufacturers instruction (Promega, Inc, Madison, WI) and normalization to growth hormone activity detected by chemiluminescent immunoassay (Nichols Institute Diagnostics, San Juan Capistrano, CA).
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71. To make plasmid p600rMIS-luc, a PCR of rat MIS phase DNA (24) with primers CMH-115 5'-CTAAAGCTTCTGCTCTGGAACCCCTGTGGCCAG-3' and CMH-116 5'-CTAAAGCTTGCGAGGGTCTGGGTGGTCCTGC-3' was subcloned into TA vector, sequenced and subcloned into the Hind III site of pA3-luc (41). Using an identical strategy, primers CMH-106 5'-GGGAA-GCTTGGCCGTCCTCCAG CCTG-3' and CMH-107 5'-CGG-AAGCTTGGGTGCTGCCAGGGGCTG-3' were used to make

human promoter construct p299hMIS-Iuc, and CMH-128 5'-GGT-ACCGGGCACTGTCCCCCAACCCCAAGG-3' and CMH-107 were used to construct p114hMIS-Iuc.

72. SRY mutation I68T was made by site directed mutagenesis (55) with primer I68T CMH-081 5'-GCGAGACCACACGGT-GAATGCGTTC-3'. PCR from mutant phage using CMH-066 and CMH-067 and subcloning into TA vector, sequencing, and subcloning into the Bam HI site of pCMV were as described for pCMV-hSRY, including the T202P design at the 3' terminus (figure 11.4A). SF-1 sense and antisense expression vectors were from K. Parker.

73. We thank D. Russell for pCMV; A. Hinck and J. Markley for pTSN53; W. Wood for pA3-Iuc and -180 RSV-Iuc; S. Fields-Berry and C. Cepko for retrovirus  $\psi$ 2-v-myc packaging cells; D. C. Page for pDP1327 and helpful advice; D. Moore for helpful advice; S. Nasser for expert assistance; R. Kingston, C. Miller, W. Walker, and M. Lachenmann for advice; S. Burley for coordinates of TBP; J. Thomas for coordinates of HMG1; D. Moore, J. Habener, D. MacLaughlin, and L. Perkins for critically reviewing the manuscript; F. Tao for experiments in figure 11.2E; R. Peters for assisting in protein purification and in experiments in figures 11.2E and 11.3A; J. Lee and C. Pabo for NMR advice; T. Havel for software and advice; G. Waneck and M. Kurian for help with mutagenesis; and D. Wiley for use of spectropolarimeter. Supported by NIH grants HD30812 (P.K.D.), GM51558 (M.A.W.), and Reproductive Endocrine Sciences Center P30HD28138 (P.K.D.).



In man, the most distal portions of Xp and Yp are “pseudoautosomal.” They undergo frequent X–Y recombination during normal male meiosis. As a result, these portions of Xp and Yp are homologous, and their inheritance is not strictly sex-linked (8–12).

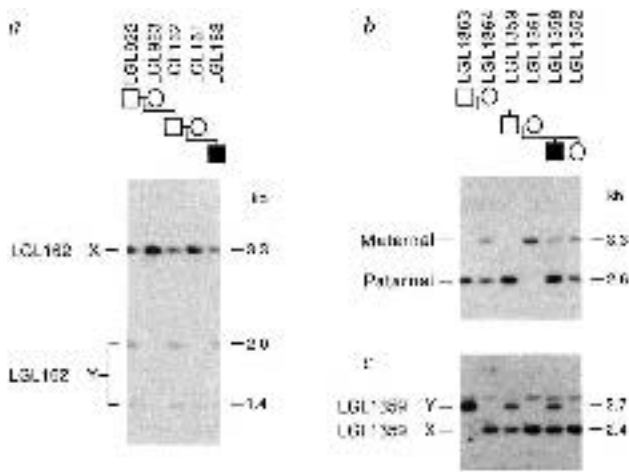
The *MIC2* gene maps proximally in the pseudoautosomal region, recombining with sex phenotype at a frequency of only 2% in male meiosis (12). Plasmid pDP1001, which detects a restriction fragment length polymorphism (RFLP) at *MIC2*, was hybridized to DNAs from the family of XX male LGL163 (figure 12.1a). The father is heterozygous; his 1.4- and 2.0-kilobase (kb) fragments must be on his Y chromosome, because that allele is present in the grandfather but not the grandmother. The XX male inherited the father’s Y allele. Densitometry reveals that the XX male has one copy of the 3.3-kb allele. This copy must be from the mother, because she is homozygous for that allele. Thus, at *MIC2*, XX male LGL163 inherited his father’s Y allele but not his father’s X allele.

The second family was typed for a *MIC2* RFLP detected by pDP1002 (figure 12.1b). XX male LGL1358 has two copies of the 2.6-kb allele, for which the father is homozygous, and one copy of the 3.3-kb allele, for which the mother is homozygous. (Compare, in figure 12.1b, the relative intensities of the 2.6- and 3.3-kb fragments in XX male LGL1358 with those in LGL1364 and LGL1362, both presumably normal heterozygotes.) A similar result was obtained with the pDP1001 RFLP: the XX male has two copies of the allele for which the father is homozygous and one copy of the allele for which the mother is homozygous (not shown).

These studies did not reveal the chromosomal origin of the two copies of *MIC2* that XX male LGL1358 received from his father. This information was obtained using an RFLP detected by pSG1, a *MIC2* complementary DNA clone (12). The father is heterozygous (figure 12.1c). His 2.7-kb fragment must be on his Y chromosome, because that allele is present in the grandfather but not the grandmother. Conversely, the father’s 2.4-kb allele must be on his X chromosome. XX male LGL1358 appears to have two

copies of the 2.4-kb allele and one copy of the 2.7-kb allele. (Compare, in figure 12.1c, the relative intensities of the 2.4- and 2.7-kb fragments in LGL1358 with those in LGL1359, a normal heterozygote.) The XX male inherited the 2.7-kb fragment from his father’s Y chromosome. The XX male presumably inherited one copy of the 2.4-kb fragment from his mother, who is homozygous for that allele. Given the results with pDP1001 and pDP1002, he must have inherited the second 2.4-kb fragment from his father’s X chromosome. That is, XX male LGL1358 inherited both his father’s X and Y alleles at *MIC2*.

The findings in these XX males have implications for the localization of the testis determining factor gene, *TDF*, on the Y chromosome. An eight-interval deletion map of the Y has been constructed from studies of Y chromosome DNA from XX males and other individuals with sex chromosome anomalies; *TDF* maps to interval 1, on Yp (3 and 13). Uncertainty regarding the order of intervals 1, 2, and 3 on Yp stems from uncertainty as to whether XX males have received terminal or internal portions of Yp (3). Given that *MIC2* recombines with sex phenotype in only 2% of normal male meioses (12), the “terminal” and “internal” models make opposite predictions as to the inheritance of *MIC2* in XX males. According to the terminal model, an XX male would receive the end of the strictly sex-linked portion of Yp from his father; he would then be very likely to inherit his father’s Y-chromosomal allele at the closely-linked *MIC2* locus (probability 98%). According to the internal model, an XX male would not receive the end of the strictly sex-linked portion of Yp; he would inherit his father’s Y-chromosomal *MIC2* allele only in the unlikely event of recombination with *MIC2* (probability 2%). That both XX males inherited their father’s Y allele at *MIC2* strongly suggests that they received terminal portions of Yp. In turn, this argues that deletion interval 1, containing *TDF*, is just proximal to the pseudoautosomal region. Similarly, that XX male LGL163 did not inherit his father’s X allele at *MIC2* suggests that the end of the strictly sex-linked portion of Xp has been lost.



**Figure 12.1**

Inheritance in two XX male families of RFLPs at *MIC2*. Squares, males; circles, females; filled squares, XX males. Both XX males carry DNA sequences derived from the strictly sex-linked portion of Yp (ref. 3 and unpublished results). Study of the paternal grandparents allows the phase of the pseudoautosomal alleles in the father to be determined. Each autoradiogram lane corresponds to the individual above that lane in the pedigree. *a*, Family 1: probe pDPI001 hybridized to *TaqI*-digested genomic DNAs. Allelic restriction fragments marking the X or Y chromosomes of LGL162, the father, are indicated. *b*, Family 2: pDPI002 hybridized to *TaqI*-digested DNAs. Allelic fragments present in father, LGL1359, or in mother, LGL1361, are indicated. *c*, Family 2: an 0.7-kb *EcoRI*-*StuI* fragment purified from plasmid pSG1 was hybridized to *MspI*-digested DNAs. Fragments that distinguish the X and Y chromosomes of LGL1359, the father, are indicated.

**Methods.** Plasmids pDPI001 and pDPI002 were subcloned from recombinant phages identified by screening a human genomic library with plasmid pSG1, a *MIC2* cDNA clone (21). Plasmid pDPI001 contains a 1.5-kb genomic *EcoRI*-*PstI* fragment and detects a *TaqI* RFLP with fragments of, in one allele, 3.3 kb and, in a second allele, 1.4 and 2.0 kb. Plasmid pDPI002 contains a 0.8-kb genomic *EcoRI* fragment. It detects an insertion/deletion RFLP (not shown) and a *TaqI* RFLP with allelic fragments of 2.6 and 3.3 kb. These clones were mapped to the region Xp22.32-pter and to the Y by hybridization to DNAs from hybrid somatic cell lines, consistent with their being derived from *MIC2*. Human genomic DNAs were prepared from leukocytes or cultured fibroblasts (22), digested with restriction endonuclease, electrophoresed on 0.7% agarose gels, and transferred (23) to nylon membrane. Human inserts purified from the plasmids were labelled with  $^{32}\text{P}$  by random-primer synthesis (24), prehybridized with an excess of sonicated human genomic DNA (25), and hybridized overnight to genomic DNA transfers at 47 °C in 50% formamide,  $5 \times \text{SSC}$  ( $1 \times \text{SSC} = 0.15 \text{ M NaCl}$ , 15 mM Sodium citrate pH 7.4), Denhardt's (0.02% Ficoll 400, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin), 1% SDS, 20 mM  $\text{NaPO}_4$  pH 6.6, 50  $\mu\text{g ml}^{-1}$  denatured salmon sperm DNA and 10% dextran sulphate. Membranes were washed three times for 15 min each at 65–70 °C in  $0.1 \times \text{SSC}$ , 0.1% SDS and exposed at –80 °C for 1–3 days with X-ray film backed by an intensifying screen.

We also followed the inheritance in these families of five other pseudoautosomal RFLPs (table 12.1). Three of these polymorphic loci, *DXYS17*, *DXYS15*, and *DXYS28*, show partial sex linkage, recombining with sex phenotype in male meiosis at frequencies of about 14%, 32% and 38%, respectively (11 and D. P. et al., in preparation). The other two loci, *DXYS20* and *DXYS14*, show no detectable sex linkage and map to the most distal portion of the pseudoautosomal region, near the telomeres of Xp and Yp (9 and 11; D. P. et al., in preparation).

All five RFLPs are informative in the first family, and at each locus the XX male inherited from father the Y but not the X allele (table 12.1). For example, the father is heterozygous for the *DXYS14* RFLP (figure 12.2a). The father's X and Y alleles are identified by their presence in, respectively, the grandmother and grandfather. XX male LGL163 inherited from his father the Y but not the X allele at *DXYS14*.

Thus, at six loci, together spanning nearly the entire pseudoautosomal portion of the sex chromosomes, XX male LGL163 inherited from father the Y but not the X alleles. These results argue that LGL163 inherited the pseudoautosomal region of his father's Y chromosome intact and unrecombined. A double crossover between two of the pseudoautosomal markers for which we tested is unlikely, given that double recombinants occur rarely if ever within the pseudoautosomal region in man (11; D. P. et al., in preparation). The results also suggest that XX male LGL163 inherited no part of the pseudoautosomal region of his father's X chromosome.

In the second family, apart from *MIC2*, one pseudoautosomal locus was informative (table 12.1). At the distal locus *DXYS14*, XX male LGL1358 inherited from his father the Y allele but not the X allele (figure 12.2b).

We propose that sex reversal in these XX males is the result of aberrant Xp–Yp exchanges (figure 12.3). XX male LGL163 appears to have inherited the entire pseudoautosomal region of his father's Y chromosome but no part of the pseudoautosomal region of his father's X chromosome. This is probably the result of a single crossover proximal to *MIC2* on Xp and proximal to *TDF* on Yp (figure 12.3a). The result is a transfer of a terminal, male-determining portion of Yp onto distal Xp, in exchange for a terminal portion of Xp, as predicted by the "X–Y interchange" model (7, 14).

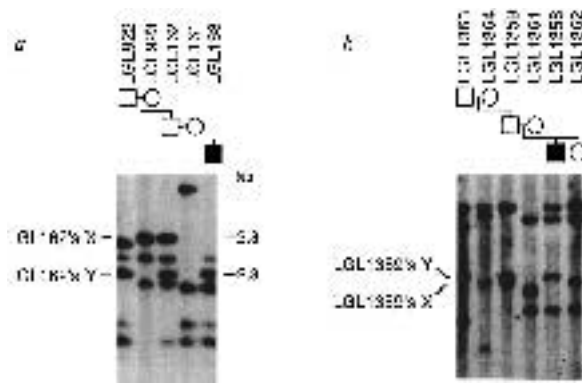
XX male LGL1358 also seems to have inherited the entire pseudoautosomal region of his father's Y chromosome. In addition, however, he inherited the proximal portion (*MIC2*) but not the distal portion (*DXYS14*) of the pseudoautosomal region of his father's X chromosome. This is probably the result of

**Table 12.1**

X- or Y-chromosomal origin of pseudoautosomal RFLP alleles transmitted from fathers to XX-male sons

Locus	Probe	Recombination with Sex Phenotype (%)	Reference	Allele from Father	
				LGL163 (XX Male 1)	LGL1358 (XX Male 2)
<i>DXYS14</i>	29C1	50	9, 11	Y	Y
<i>DXYS20</i>	pDP230	50	Page et al. (in preparation)	Y	NI
<i>DXYS28</i>	pDP411a	38	Page et al. (in preparation)	Y	NI
<i>DXYS15</i>	113D	32	10, 11	Y	NI
<i>DXYS17</i>	601	14	11	Y	NI
<i>MIC2</i>	pSG1 pDP1001 pDP1002	2	12	Y	X and Y

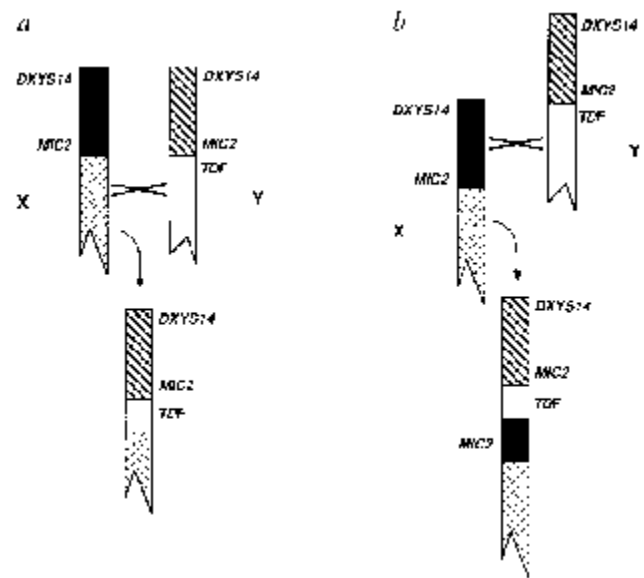
DNA probes detecting pseudoautosomal RFLPs were hybridized to *TaqI*, *MspI*, or *EcoRI*-digested DNAs from two XX males, their parents, and paternal grandparents. NI, not informative.

**Figure 12.2**

Inheritance in two XX-male families of RFLPs at *DXYS14*. *a*, Family 1: probe 29C1 was hybridized at 42 °C to *TaqI*-digested genomic DNAs. After washing at 60 °C, the transfer membrane was exposed with X-ray film for 3 days. Probe 29C1 detects a polymorphic family of related sequences, resulting in complex hybridization but simple, single-locus inheritance; a typical allele comprises a collection of restriction fragments (9, 11). Some of the allelic restriction fragments that distinguish the X and Y chromosomes of LGL162, the father, are indicated. *b*, Family 2: 29C1 hybridized to *EcoRI*-digested DNAs. Some of the allelic restriction fragments marking the X and Y chromosomes of LGL1359, the father, are indicated.

a single crossover in the pseudoautosomal region (between *MIC2* and *DXYS14*) on Xp and proximal to *TDF* on Yp (figure 12.3b), as has been hypothesized to occur in some XX males (14).

X–Y exchanges like that in figure 12.3b produce XX males with two copies of all strictly X-linked loci. In contrast, exchanges of the sort seen in LGL163 (figure 12.3a) might produce XX males with a single copy of strictly sex-linked loci on distal Xp. This may explain why some XX males express their fathers' alleles for *Xg*, a dominant, X-linked marker on distal Xp, whereas most, including LGL163, do not (1, 15). Exchanges like that in figure 12.3b would yield *TDF*-bearing X chromosomes whose length is greater than that of normal X chromosomes. Depending on the positions of

**Figure 12.3**

Genesis of *TDF*-bearing X chromosomes in XX males by single crossovers between Xp and Yp during or prior to paternal meiosis. Differentially shaded regions depict the pseudoautosomal (black and striped) and strictly sex-linked (stippled and white) portions of Xp and Yp. On Yp, the point of crossing over is proximal to *TDF*, which in turn is proximal to the pseudoautosomal region. On Xp, crossing over can occur either *a*, proximal to *MIC2*, perhaps in the X-specific region, or *b*, distal to *MIC2*, in the pseudoautosomal region. Unequal crossing-over depicted in *b* produces an X–Y interchange product carrying two copies of *MIC2*.

Xp and Yp breakpoints, exchanges like that in figure 12.3a might yield abnormally long or short TDF-bearing X chromosomes. Such alterations may explain the observation that, in many XX males, one of the two X chromosomes seems to be abnormally long (16) or has an altered high-resolution banding pattern (17).

Distal portions of Xp and Yp pair during male meiosis (18, 19). Does this pairing contribute to the rather high frequency (1 in 20,000 males [1]) at which XX males occur? Various mechanisms can be envisaged. First, homologous X–Y recombination initiated in the pseudoautosomal region might, on occasion, give rise to branch migration into the strictly sex-linked portions of the sex chromosomes, with resolution proximal to *TDF*. This seems unlikely, however, because branch migration seems to require that recombining chromosomes have very similar DNA sequences; the Yp-specific DNA sequences that XX males inherit are not homologous to Xp.

A second possibility is more likely. The Xp–Yp synaptonemal complex extends far beyond the pseudoautosomal region, involving the distal quarter of Xp and virtually all of Yp (20). This synapsis might occasionally produce exchanges between the strictly sex-linked portions of the X and Y chromosomes. These exchanges may or may not be legitimate recombination events occurring at sites of limited X–Y homology. To account for XX males in whom the point of recombination on X is pseudoautosomal, one would have to suppose that Xp–Yp synapsis is compatible with the pseudoautosomal portions of X and Y chromosomes being grossly out of register. Perhaps the pseudoautosomal regions of the X and Y chromosomes, so highly recombinogenic in male meiosis, retain this property when misaligned.

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Significant progress has been made toward defining the role of androgens in sexual differentiation and development in the past 25 years. Jost in pioneer experiments on rabbit fetuses demonstrated that female organogenesis, that is, Mullerian stimulation and Wolffian inhibition, will occur in the absence of the gonads (1). Male sexual differentiation is imposed upon the natural tendency of the fetus toward femaleness. Normal male sexual differentiation requires the secretion of two factors by the testes. At a critical period of embryogenesis, testosterone, secreted by the Leydig cells, stimulates differentiation of the Wolffian anlage to the epididymis, vas deferens, and seminal vesicles, and differentiation of the urogenital sinus, urogenital tubercle, and urogenital swellings to form the male external genitalia and prostate. Mullerian inhibition, however, is not mediated by androgens, but results from the action of Mullerian inhibiting factor, probably secreted by the seminiferous tubules (2).

Within the last 10 years, investigators have shown that testosterone may act as a prehormone, that is, in specific androgen-dependent target areas, it is converted by the microsomal enzyme  $\Delta^4$ -steroid 5 $\alpha$ -reductase to form 5 $\alpha$ -dihydrotestosterone, a more potent androgen (3). It has been demonstrated in human fetuses that, at the time of sexual differentiation in utero, dihydrotestosterone formation occurs in the urogenital sinus, urogenital tubercle, and urogenital swellings, but dihydrotestosterone formation does not occur in the Wolffian anlage until after differentiation has occurred (4).

The data suggest that there may be at least two androgens involved in sexual differentiation, with selective roles for testosterone and dihydrotestosterone during embryogenesis. The male pseudohermaphrodites described below define the necessity for dihydrotestosterone during embryogenesis and delineate the actions of testosterone and dihydrotestosterone in sexual differentiation and development.

To date we have found 13 families with 24 male pseudohermaphrodites, in the village of Salinas in the Dominican Republic (5). The affected males (46 XY) (6) are born with marked ambiguity of the external

genitalia, and before the disorder became obvious to the community were raised as girls. At birth, they have bilateral testes presenting as inguinal or labial masses, a labial-like scrotum, a urogenital sinus with a blind vaginal pouch, and a clitoral-like phallus. No Mullerian structures are present.

At puberty, their voice deepens and they develop a typical male phenotype with a substantial increase in muscle mass; there is no breast enlargement. The phallus enlarges to become a functional penis, and the change is so striking that these individuals are referred to by the townspeople as “guevedoces”—penis at 12 (years of age). The scrotum becomes rugated and hyperpigmented, the testes descend from the inguinal canal, and there is an ejaculate. The prostate remains small, beard growth is scanty, and there is no temporal recession of the hairline or acne. Psychosexual orientation is unequivocally male. Testicular biopsy demonstrates complete spermatogenesis, with normal Leydig cells. There is a normal epididymis and vas deferens.

Thus, at birth the defect is limited to incomplete differentiation of the male external genitalia; masculinization of the internal structures is normal. At puberty, virilization occurs with the exception of a scanty or absent beard, lack of temporal recession of hairline, and a small to absent prostate.

Because of the virilization at puberty, and despite marked ambiguity of the external genitalia at birth, we hypothesized that the affected individuals would not have a disorder of testosterone biosynthesis. The male puberty without breast development and with complete spermatogenesis also precludes a defect due to impaired androgen action. We proposed, therefore, that the abnormality was due most likely to a defect in the metabolism of testosterone at the target issue, that is, biotransformation of testosterone to 5 $\alpha$ -dihydrotestosterone by the enzyme  $\Delta^4$ -steroid 5 $\alpha$ -reductase (7).

To define a defect in 5 $\alpha$ -reductase activity, plasma testosterone and 5 $\alpha$ -dihydrotestosterone were measured in four affected males by a double isotope derivative technique (8). In the affected males the plasma

testosterone concentration ranged from 470 to 960 ng per 100 ml, which was within the normal male range of 300 to 1200 ng per 100 ml. However, dihydrotestosterone concentrations were 16, 17, 21, and 29 ng per 100 ml, which were below the normal male range of 40 to 80 ng per 100 ml. The ratio of plasma testosterone to dihydrotestosterone in normal males was approximately 14/1, and in the affected males it was approximately 40/1.

In two affected males, the percentage conversion of testosterone to  $5\alpha$ -dihydrotestosterone was measured during continuous infusion of radioactive testosterone (9). The percentage conversion was 0.48 and 0.85, and was approximately one-sixth of the reported normal range of 3.5 to 7.0.

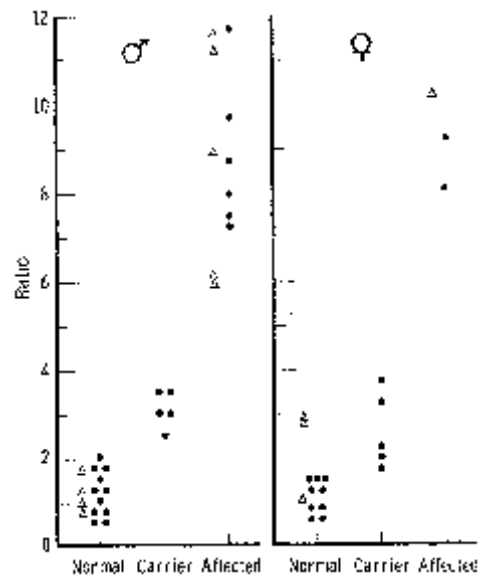
Reduction of the double bond between rings A and B of neutral steroids, such as testosterone, is catalyzed in the liver by  $\Delta^4$ -steroid  $5\beta$ -reductase (or reductases) localized to the cytosol, and the  $\Delta^4$ -steroid  $5\alpha$ -reductase (or reductases) of the membranes of the endoplasmic reticulum. However, a substantial fraction of testosterone is metabolized in extrahepatic tissue (10), and most, if not all, proceeds to the *trans* or  $5\alpha$  configuration (11).

In normal, affected, and obligate carriers, we measured the  $C_{19,11}$ -deoxysteroids—that is, the 17-ketosteroid metabolites  $3\alpha,5\beta$ -etiocholanolone and  $3\alpha,5\alpha$ -androsterone, and the  $17\beta$ -hydroxy metabolites  $3\alpha,5\beta$ -etiocholanediol and  $3\alpha,5\alpha$ -androstenediol.

In both postpubertal normal and affected subjects, the urinary 17-ketosteroids  $3\alpha,5\alpha$ -androsterone and  $3\alpha,5\beta$ -etiocholanolone were fractionated by an isotope dilution technique with the use of  $\beta$ -glucuronidase hydrolysis. Tritium-labeled androsterone and etiocholanolone were added to the urine samples (30 ml) before hydrolysis to correct for procedural losses. The steroids were purified by paper chromatography, and quantitated by the Zimmerman reaction.

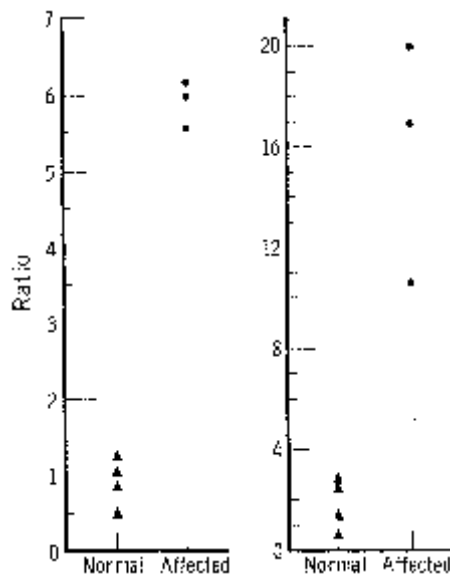
After glucuronidase hydrolysis of the urines of 11 normal males, the  $5\beta/5\alpha$  ratios of the urinary 17-ketosteroids etiocholanolone and androsterone, were 0.5 to 2.0 with a mean of 1.2 (figure 13.1). In six affected males, the mean ratio was 8.5, with a range of 7.3 to 11.8. Five obligate carriers (fathers) showed an intermediate range of 2.5 to 4.3, with a mean ratio of 3.5. Nine normal females had a ratio of 0.7 to 1.6, with a mean ratio of 1.1. Two phenotypically normal females, from a family with three affected males, had ratios of 8.3 and 9.6, and are homozygous for the condition. In five obligate carriers (mothers), the ratio was 1.8 to 3.7, with a mean of 2.5.

The urinary  $17\beta$ -hydroxysteroid glucuronides  $3\alpha,5\alpha$ -androstenediol and  $3\alpha,5\beta$ -etiocholanediol were determined by a double isotope derivative procedure on 10-ml samples of urine, with the use of  $^{14}\text{C}$ -labeled ste-



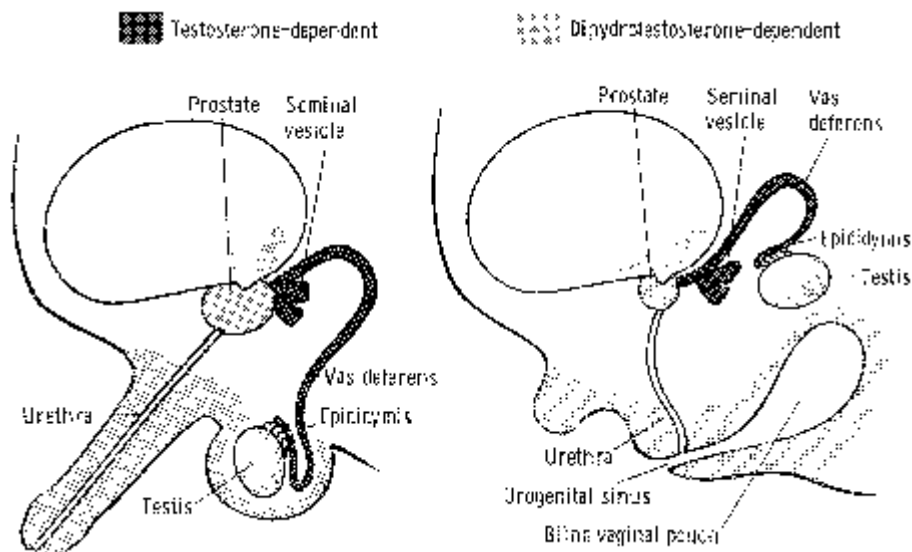
**Figure 13.1**

Ratio of endogenous urinary  $3\alpha,5\beta$ -etiocholanolone to  $3\alpha,5\alpha$ -androsterone (●) and  $3\alpha,5\beta$ -etiocholanediol to  $3\alpha,5\alpha$ -androstenediol (Δ) in males and females—that is, normals, carriers, and affected.



**Figure 13.2**

Ratio of radioactive urinary (left)  $3\alpha,5\beta$ -etiocholanolone to  $3\alpha,5\alpha$ -androsterone and (right)  $3\alpha,5\beta$ -etiocholanediol to  $3\alpha,5\alpha$ -androstenediol after  $[^3\text{H}]$ testosterone infusion in normal (▲) and affected (●) males.



**Figure 13.3**

Illustration of the hypothesis for the role of testosterone and dihydrotestosterone in male sexual differentiation in utero.

roids to correct for procedural losses and tritium-labeled acetic anhydride (25 mc/mole) to measure mass.

The  $5\beta/5\alpha$  ratio of the urinary glucuronides of etiocholanol and androstanediol in four normal males and three normal females ranged from 0.8 to 3.0. In five affected males, the ratio was 6.0 to 11.8; in one phenotypically normal female with an abnormal  $5\beta/5\alpha$  ratio of urinary 17-ketosteroids, the ratio was 10.2.

[ $^3\text{H}$ ]Testosterone was infused into four normal and three affected males (figure 13.2). After the urine was treated with glucuronidase and hydrolyzed with hot acid, the  $5\beta/5\alpha$  ratios of etiocholanolone to androsterone in the normal males ranged from 0.5 to 1.13 with a mean of 0.87 and from 5.5 to 6.4 in the affected males. In normals, the urinary  $5\beta/5\alpha$  ratio of etiocholanol to androstanediol was 0.46 to 2.7; and in the affected males, 11.0 to 20.

Thus, analysis of the  $5\beta$ - and  $5\alpha$ -11-deoxy  $\text{C}_{19}$ -steroid metabolites (both endogenous and radioactive) revealed a marked decrease in  $5\alpha$  reduced metabolites in affected males (12), and an intermediate decrease in obligate carriers.

The above studies demonstrate a defect in  $5\alpha$  reduction resulting in the decreased conversion of testosterone to dihydrotestosterone. Whether the biochemical error involves the synthesis, structure, or metabolism of the enzyme  $\Delta^4$ -steroid  $5\alpha$ -reductase is not known.

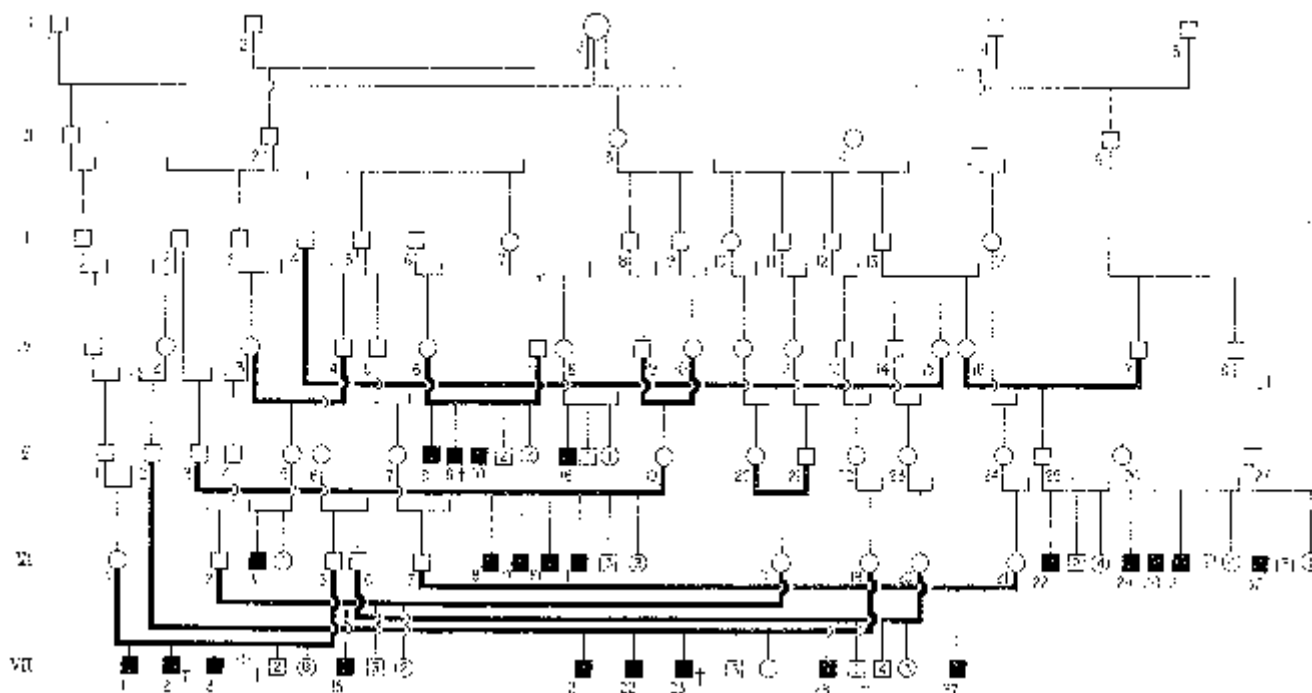
From the clinical presentation of ambiguous external genitalia with normal male internal structures and the biochemical data demonstrating  $\Delta^4$ -steroid  $5\alpha$ -reductase deficiency with decreased dihydrotestosterone formation, we hypothesize that during embryo-

genesis and again at puberty, both testosterone and dihydrotestosterone are necessary for complete male external differentiation and development (figure 13.3). Testosterone secreted in utero by the testes acts directly on the Wolffian ducts to cause differentiation to the vas deferens, epididymis, and seminal vesicles; but in the urogenital sinus and urogenital tubercle, testosterone functions as a prehormone, where its conversion to dihydrotestosterone results in differentiation of the external genitalia and prostate.

The anabolic events at puberty, in particular the increase in muscle mass, the growth of the phallus and scrotum, and the voice change, appear to be mediated by testosterone and occur in the affected males (11, 13). Prostate growth, facial hair, temporal recession of the hairline, and acne do not occur and appear to be mediated by dihydrotestosterone (14).

Psychosexual orientation (postpubertal) is male, and this is of considerable interest, since the sex of rearing in 18 of the affected males was female. Despite the sex of rearing, the affected were able to change gender identity at the time of puberty. They consider themselves as males and have a libido directed toward the opposite sex. Thus, male sex drive appears to be testosterone related and not dihydrotestosterone related (15), and the sex of rearing as female, appears to have a lesser role in the presence of two masculinizing events—testosterone exposure in utero and again at puberty with the development of a male phenotype.

Salinas (population 4300), is a geographic isolate 150 miles (1 mile = 1.6 km) west of Santo Domingo, Dominican Republic. Within the village, the frequency of normal to affected males is approximately 90/1. The



**Figure 13.4**

Pedigree illustrating common ancestry in 12 of the 13 families, and transmission of the defect for male pseudohermaphroditism through seven generations.

affected males range in age from  $1\frac{1}{2}$  to 60 years. Figure 13.4 is a pedigree illustrating the transmission of the defect through seven generations. In 12 of the 13 families, one line of descent can be traced back to Altargracia Carrasco I-3, and in seven of the families both lines can be traced to the same woman.

The isolation of the town, together with the pedigree demonstrating common ancestry, suggests that the increase in gene frequency is a consequence of genetic drift—a founder effect. However, the heterozygotes may have a selective advantage which contributes to gene frequency. The increased incidence of consanguinity, the presence of the biochemical defect in both sexes, and phenotypically normal carriers of both sexes with an intermediate biochemical abnormality, support autosomal recessive inheritance.

In summary, we have described an inherited form of male pseudohermaphroditism secondary to  $\Delta^4$ -steroid  $5\alpha$ -reductase deficiency, elucidating the action of dihydrotestosterone in the development of the male external genitalia in utero. This entity also demonstrates for the first time an inherited disorder of steroid metabolism.

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16. We thank T. Huang and F. Guzman for technical assistance. Aided in part by NIH training grant AM 5250; PHS research career award K6-AM 14, 241-11 (to R.E.P.) and PHS clinical research center grant RR 47.

S. F. Ahmed, A. Cheng, L. Dovey, J. R. Hawkins, H. Martin, J. Rowland, N. Shimura, A. D. Tait, and I. A. Hughes (2000) Phenotypic features, androgen receptor binding, and mutational analysis in 278 clinical cases reported as androgen insensitivity syndrome. *Journal of Clinical Endocrinology and Metabolism* 85: 658–665.

Defects of the androgen receptor (AR) cause the androgen insensitivity syndrome (AIS), an X-linked disorder in 46XY individuals with normal androgen production and metabolism. AIS is estimated to be present in 1:20,000–64,000 male births, and variable phenotypic expression has allowed the classification of AIS into complete (CAIS) and partial forms (PAIS), as well as a rare group of phenotypically normal men with azoospermia (1, 2). While individuals with CAIS have female external genitalia, affected cases of PAIS have variable ambiguity of the genitalia and often undergo extensive reconstructive surgery. If reared as girls, both groups also undergo gonadectomy to eliminate the risk of gonadal malignancy (3). Demonstration of normal testosterone and dihydrotestosterone production is necessary with PAIS to exclude defects in testosterone biosynthesis and 5 $\alpha$ -reductase deficiency (4, 5). AR binding can be assessed, in vitro, in cultured genital skin fibroblasts (4), but the parallel development of assays in different laboratories has led to a confusing unstandardized form of nomenclature (1). A diverse range of AR-binding defects can be demonstrated in some, but not all, cases of AIS (4–8).

The gene encoding AR is localized to Xq11–12 (9) and cloning of the human AR complementary DNA has allowed characterization of the molecular defects responsible for AIS. A variety of different strategies for mutational screening of the AR gene have revealed over 300 mutations in AIS (10, 11). It is, however, unclear whether there is a relationship between the site and type of mutation and the abnormality in androgen binding. In addition, prenatal diagnosis, as well as decisions about sex of rearing, is hindered because of the clinical heterogeneity of phenotype for a given mutation within the same family. Modifications of the Prader classification of genital ambiguity have been used to classify the male under-masculinization associated with AIS (1). It is, however, possible that a decision about sex of rearing influences the assessment of phenotype based on this kind of classification and there is a need for more objective standardization of this assessment. Large cohort studies of AIS have rarely been reported, but over the last decade the AIS component

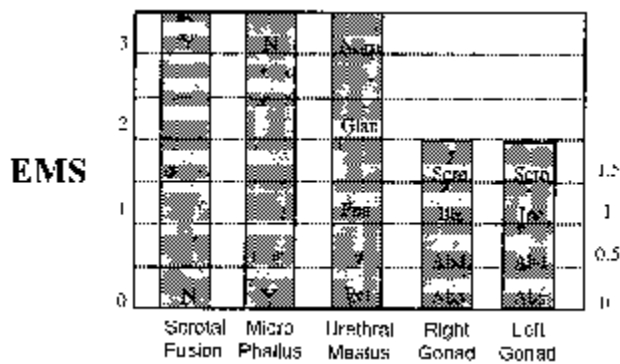
of a United Kingdom ambiguous genitalia and intersex database held in Cambridge is now large enough to provide a comprehensive review of a range of features associated with AIS.

Here, we describe a large cohort of patients with male under-masculinization in whom the clinical diagnosis of AIS can be confirmed in a significant number of cases by a combination of androgen-binding studies and mutational analysis. Some correlation between the phenotypic features and the abnormalities identified on mutational analysis of the AR gene is apparent, but the phenotypic heterogeneity among clinically diagnosed cases of AIS emphasizes the need for appropriate comprehensive evaluation of male under-masculinization. It is clear that while a phenotype consistent with PAIS is a common cause of male under-masculinization, specific mutations involving the X-linked AR gene account for only a proportion of PAIS cases.

### Subjects and Methods

Detailed clinical information on every case notified to the database held in Cambridge was collected via a questionnaire, and the diagnosis was entered as reported by the clinician. Among the 816 entries recorded by January 1999 there were 105 clinically diagnosed cases of CAIS and 173 cases of PAIS. In every reported case of AIS where there were sufficient clinical data, a masculinization score was created by assessing specific clinical features, as detailed in figure 14.1. A maximum score of 12 represented normal masculinization.

When genital skin was obtained at time of surgery, androgen-binding studies were performed on cultured genital skin fibroblasts as reported previously (5). Parameters measured included binding capacity ( $B_{max}$ ) that reflects receptor concentration and receptor-binding affinity ( $K_d$ ). Binding was classified as either zero, abnormal, or normal based on an updated reference range for binding parameters using circumcision samples from normal individuals ( $n = 23$ ) (unpublished data). For our laboratory, the range of  $B_{max}$  and  $K_d$  in normal genital skin fibroblasts is



**Figure 14.1**

Criteria for the masculinization score. Y, yes; N, no; Norm, normal; Blan, glandular; Pen, penile; Per, perineal; Scro, scrotal; Ing, inguinal; Abd, abdominal; Abs, absent.

greater than  $300 \times 10^{-18}/\mu\text{g DNA}$  and  $0.8-1.7 \times 10^{-10}$  M, respectively.

Mutation screening of all eight exons of the AR gene was performed as described previously using single-strand conformational polymorphism analysis (SSCA), followed by direct DNA sequencing of any PCR products showing abnormal conformation on SSCA (12). AR-binding studies, as well as mutational analyses, were performed in those cases where other causes of male under-masculinization could be excluded on the basis of the clinical and biochemical data provided. Details of some of the mutational analyses have been reported previously (12-16). Histological details were obtained from the reporting clinician in those cases where either testicular biopsy or gonadectomy was performed. The Wilcoxon signed rank test and the  $\chi^2$  test were performed to enable comparison between groups.

## Results

### Subjects and Family History

One hundred five cases with a clinical diagnosis of CAIS and a median age of 13.2 yr (10th and 90th percentiles, 3.8 and 26.8) were identified in the database. Their median age at presentation was 1 yr (P10, P90: 0.1, 10.4). One hundred seventy-three cases with a clinical diagnosis of PAIS and a median age of 8.0 yr (P10, P90: 3.0, 2.5) were also identified. In the 81 cases of CAIS where family history was available, 52 cases had a family history of AIS, whereas 29 did not. Family history of PAIS was positive in only 31 cases, negative in 116 cases, and unknown in 26 cases.

### Mode of Presentation

All cases of PAIS presented within 1 month of birth with genital ambiguity. The mode of presentation was unknown in 33 cases of CAIS. Twenty-eight of 72 cases (39%) of CAIS presented with bilateral hernia, 20

(28%) presented with unilateral hernia, 15 (21%) presented with a positive family history, 4 (6%) presented with an amniocentesis-karyotype mismatch with phenotypic sex, and 4 (6%) presented with primary amenorrhea.

### Gonadal Position, Histology, and Outcome

The position of the gonads was unrecorded in 25 cases of CAIS. Twenty-nine of 80 (36%) cases of CAIS had bilateral abdominal testes, whereas in 33 (41%) cases the gonads were bilaterally palpable in the labioscrotal folds or in the inguinal region. In 18 cases, they were abdominal on one side but descended on the other. Gonadal position was unknown in eight cases of PAIS. Bilateral abdominal testes were present in 21 of 165 cases (13%) of PAIS; 128 (77%) cases had testes that were bilaterally descended or in the inguinal region, and in 13 (8%) cases they were descended on one side but abdominal on the other. In three cases of PAIS, testes were absent on one side.

Eighty-one of 105 cases of CAIS were reported to have had bilateral gonadectomy. In 54 (66%) cases, gonadectomy had been performed before puberty, whereas 23 (29%) had postpubertal gonadectomy. In four cases, the timing was unknown. The median age at presentation of girls who had postpubertal gonadectomy was 14 yr (P10, P90: 0.1, 18). Gonadectomy had been performed in 46 cases of PAIS; in 18 cases this was performed before puberty, whereas in 4 cases it was performed after puberty. The sex of rearing in these four cases had been female. The timing of gonadectomy was unknown in the remaining 24 cases of PAIS.

Testicular histology, available in 65 of 77 cases of CAIS who had gonadectomy, was reported as normal testes in 59 (91%). In three cases, the testes were described as atrophic, in two cases there was evidence of marked fibrous tissue deposition, and in one case bilateral hamartoma were described. Testicular histology reports were available in 56 of 173 cases of PAIS and were reported as normal in 53 and abnormal in 3, with evidence of testicular atrophy. A more detailed analysis of gonadal histology in this series of AIS cases is the subject of a separate publication.

### Biochemical Investigations

Biochemical investigations were performed in 50 of the total 105 cases of CAIS. Of this subset, baseline sex steroid concentrations were determined in 49 of 50 (98%) cases, an human CG (HCG) stimulation test was performed in 26 (52%) cases, baseline gonadotrophins with or without GnRH stimulation test in 27 (54%) cases, and a urinary steroid profile in 6 (12%) cases. Biochemical investigations were reported in 159 of 173 cases of PAIS. Baseline sex steroids were measured in

**Table 14.1**

Details of AR-binding studies in clinically diagnosed subjects with CAIS and PAIS

	Low K <sub>d</sub> Low B <sub>max</sub>	Normal K <sub>d</sub> Low B <sub>max</sub>	Low K <sub>d</sub> Normal B <sub>max</sub>	High K <sub>d</sub> Low B <sub>max</sub>	High K <sub>d</sub> Normal B <sub>max</sub>	Normal K <sub>d</sub> Normal B <sub>max</sub>
<i>CAIS</i>						
Number of subjects	1	3	0	3	8	7
Median K <sub>d</sub> (range) ( $\times 10^{-10}$ M)	0.7	1.4 (1.2–1.4)	—	5.9 (5.6–7)	2.3 (2–18.6)	1.1 (0.9–1.7)
Median B <sub>max</sub> (range) ( $\times 10^{-18}$ moles/ $\mu$ g DNA)	100.2	264.6 (127.9–270)	—	209.2 (209.2–287.9)	726 (578–1731)	561 (351–1679)
<i>PAIS</i>						
Number of subjects	1	4	4	4	21	74
Median K <sub>d</sub> (range) ( $\times 10^{-10}$ M)	0.5	1.1 (0.9–1.2)	0.6 (0.6–0.7)	3.3 (2–8.6)	3 (1.8–12.6)	1.1 (0.8–1.6)
Median B <sub>max</sub> (range) ( $\times 10^{-18}$ moles/ $\mu$ g DNA)	141.3	146.4 (120–216)	1017.5 (428–1747)	188.4 (162.8–282.7)	846 (310–2057)	775 (308–1780)

<sup>a</sup>Twenty-nine cases of CAIS and six cases of PAIS had zero binding.

152 (96%) cases, an HCG stimulation was performed in 138 (87%) cases, baseline gonadotrophins with or without GnRH stimulation in 130 (82%) cases, and a urinary steroid profile in 52 (33%) cases.

#### Androgen-Binding Assays

AR-binding assays were performed in 51 cases of CAIS and 114 cases of PAIS. Details of the results are presented in table 14.1. For the CAIS group, 29 of 51 (57%) cases had zero binding, 15 (29%) cases had abnormal binding, and 7 (14%) cases had normal binding. Only 6 cases of PAIS had zero binding, 34 of 114 (30%) cases had abnormal binding, and 74 (64%) cases had normal binding.

#### Mutational Analysis

Mutational analysis of the AR gene was performed in 69 of 99 index cases of CAIS and 43 of 173 index cases of PAIS. Analysis revealed 48 different mutations in 57 (83%) cases of CAIS (table 14.2) and 10 different mutations in 12 (28%) cases of PAIS (table 14.3). The 12 cases of CAIS and 31 cases of PAIS who did not reveal any abnormalities on SSCA remained mutation negative on direct gene sequencing. The AR gene abnormalities identified in CAIS comprised 33 substitutions, one complete deletion, nine partial deletions, two insertions, one duplication, and two splice defects where the precise mutation has not been identified. The effect of these mutations is detailed in table 14.2. Twenty-two of these mutations were deemed novel as they were not present in the AR database (11). In three cases of CAIS, more than one mutation was identified in the AR gene. In PAIS, 9 of the 10 mutations were substitutions. In addition, there was a shortened length of 12 polyglutamine repeats in exon A in three cases of PAIS who also had a mutation in exon E; these three cases were later found to be related to each other (table 14.3). In three instances, two mutations (double mu-

tant alleles) were encountered in one case. An identical mutation was found in a pair of unrelated cases of CAIS (C43) and PAIS (P10).

#### Masculinization Scores and Sex of Rearing

All children with CAIS were raised as girls. For PAIS, 112 children were raised as boys and 51 raised as girls. The sex of rearing was unknown in 10 cases. The median masculinization score of 3 (P10, P90: 2.0, 7.5) was higher in the group reared as boys than in the group of girls who had a median masculinization score of 2.5 (P10, P90: 1.0, 6.0). However, there was no significant difference between the scores on  $\chi^2$  analysis. There was a considerable degree of overlap for the masculinization score between the two sexes of rearing as shown in figure 14.2.

#### Relationships between AR Binding and AR Gene Mutational Analysis

Thirteen of the 15 cases of CAIS who had abnormal binding had mutational studies and they were positive in 11 (85%) cases. Nineteen of the 29 CAIS cases with zero binding had mutation studies, with a positive result in 17 (89%) cases. In the PAIS group, mutational analysis was performed in 17 cases with abnormal binding, with a positive yield in 9 (53%) cases. Analysis in three PAIS cases with zero binding did not reveal any mutations. Three cases of CAIS with normal binding had mutational analysis, with a positive yield in one case. In 47 cases of CAIS, binding studies were not done and the positive yield in the cases that had mutational analysis was 23 of 26 (88%). Only two cases of PAIS with normal binding had mutational analysis because of positive family history, and a mutation was found in one case. Four cases of PAIS with no binding studies had mutational analysis because of positive family history, and the mutation yield was two (50%).

**Table 14.2**  
Details of AR gene mutations and Ar-binding studies in mutation-positive cases of CAIS

ID	Exon	Mutation Type	Base Change	Codon	Change	$K_d$ ( $\times 10^{-10}$ M)	$B_{max}$ ( $\times 10^{-18}$ moles/ $\mu$ g DNA)
C1	A–H	Deletion			Complete AR deletion		Zero
C2	A	Deletion	–A	127	Frameshift		
C3	A	Deletion	–A	127	Frameshift		Zero
C4	A	Insertion	+ATCC	202	Frameshift		Zero
C5 <sup>a</sup>	A	Deletion	–G	208	Frameshift		Zero
C6	A	Substitution	GGA-TGA	371	Gly-Stop		Zero
C7	A	Deletion	–C	461	Frameshift		Zero
C8 <sup>a,b</sup>	A	Deletion		483–492	Frameshift	0.7100.2	
C9 <sup>a,b</sup>	A	Substitution	GGC-AGC	498	Gly-Ser	0.8	241.9
C10 <sup>a</sup>	B	Duplication		Exon B	Frameshift		
C11	B	Deletion		582	Phe Del	1.1	1679.8
C12	IVS2	Unidentified			Splice Site		Zero
C13	IVS2	Unidentified			Splice Site		
C14 <sup>a</sup>	C	Substitution	CGT-CCT	615	Arg-Pro	2.5	1061.4
C15	C	Deletion		Exon C	Exon C Del		Zero
C16	C	Deletion		Exon C	Exon C Del		
C17	IVS3	Substitution	GGT-GAT		Splice Site	0.9	1541
C18	IVS3	Substitution	GGT-GAT		Splice Site		
C19 <sup>a</sup>	D	Substitution	GGA-TGA	688	Gly-Stop		Zero
C20	D	Deletion		692	Asn Del	15.7	1407
C21 <sup>a</sup>	D	Substitution	TTG-ATG	700	Leu-Met		Zero
C22 <sup>a</sup>	D	Substitution	CTC-TTC	701	Leu-Phe		
C23 <sup>a</sup>	D	Substitution	AGC-TGC	703	Ser-Cys		
C24 <sup>a</sup>	D	Substitution	AGA-ACA	710	Arg-Thr		Zero
C25 <sup>a</sup>	D	Substitution	CCT-TCT	723	Pro-Ser	18.6	712
C26 <sup>a</sup>	E	Substitution	GGC-GAT	724	Gly-Asp		Zero
C27 <sup>a</sup>	E	Substitution	GGC-AGC	724	Gly-Ser		Zero
C28	E	Substitution	GGC-GAC	750	Gly-Asp		Zero
C29	E	Substitution	GGT-GAT	750	Gly-Asp		
C30	E	Substitution	TTA-TTC	762	Leu-Phe		Zero
C31	E	Substitution	GCC-ACC	765	Ala-Thr		Zero
C32	E	Substitution	GCC-ACC	765	Ala-Thr		
C33	E	Substitution	GCC-ACC	765	Ala-Thr		
C34	E	Substitution	GCC-ACC	765	Ala-Thr		
C35 <sup>a</sup>	E	Deletion	CCTG-CCG	766	Frameshift		
C36 <sup>a</sup>	E	Deletion	CCTG-CCG	766	Frameshift		
C37	E	Substitution	CCT-TCT	766	Pro-Ser		
C38 <sup>a</sup>	E	Substitution	CTG-CCG	768	Leu-Pro		
C39	F	Substitution	CGC-CAC	774	Arg-His		
C40	F	Substitution	CGC-CAC	774	Arg-His		Zero
C41	F	Substitution	CGC-CAC	774	Arg-His	1.4	270
C42	F	Substitution	CGG-TGG	779	Arg-Trp		
C43 <sup>a</sup>	F	Substitution	ATG-ATA	780	Met-Ile	5.6	287.9
C44	G	Substitution	CGA-TGA	831	Arg-Stop		
C45	G	Substitution	CGA-CAA	831	Arg-Gln		
C46	G	Insertion	AAT-AAAT	848	Frameshift		Zero
C47	G	Substitution	CGC-TGC	855	Arg-Cys		Zero
C48	G	Substitution	CGC-TGC	855	Arg-Cys	2.1	578.2
C49 <sup>a,b</sup>	G	Substitution	TTC-TTG	856	Phe-Leu		
C50	G	Substitution	GAC-AAC	864	Asp-Asn		
C51	G	Substitution	GAC-GGT	864	Asp-Gly		Zero

**Table 14.2**  
(continued)

ID	Exon	Mutation Type	Base Change	Codon	Change	$K_d$ ( $\times 10^{-10}$ M)	$B_{max}$ ( $\times 10^{-18}$ moles/ $\mu$ g DNA)
C52	G	Substitution	GTG-ATG	866	Val-Met		
C53	G	Substitution	CTG-ATG	866	Val-Met		Zero
C54	G	Substitution	CTG-ATG	866	Val-Met		Zero
C55 <sup>a,b</sup>	G	Substitution	TCC-CCC	865	Ser-Pro		
C56 <sup>b</sup>	H	Substitution	CTA-GTA	881	Leu-Val	7	53.2
C57 <sup>b</sup>	H	Substitution	GTG-ATG	889	Val-Met	1.4	264.6
C58	H	Substitution	CTT-TTT	907	Leu-Phe	16.6	708
C59 <sup>a</sup>	H	Substitution	CAC-CGC	917	His-Arg		

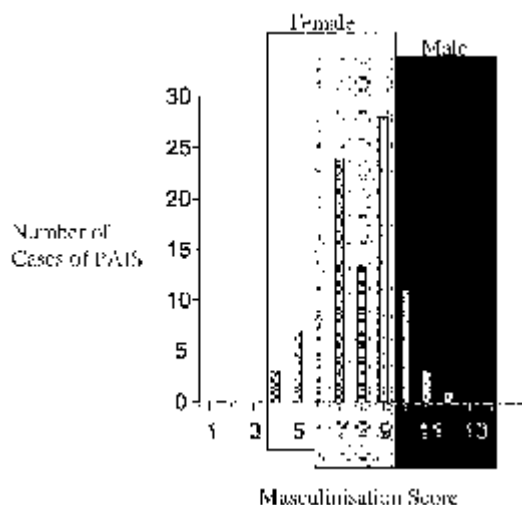
<sup>a</sup>Denotes mutations not present in the AR database (11).<sup>b</sup>The three cases that had two mutations each.**Table 14.3**  
Details of AR gene mutations and AR-binding studies in mutation positive cases of PAIS

ID	Exon	Mutation Type	Base Change	Codon	Change	$K_d$ ( $\times 10^{-10}$ M)	$B_{max}$ ( $\times 10^{-18}$ moles/ $\mu$ g DNA)
P1 <sup>a</sup>	A					2.2	1850
P2 <sup>a</sup>	A					3.0	1992
P3 <sup>a</sup>	A						
P4	C	Substitution	AGG-AAG	608	Arg-Lys	5.5	456
P5	D	Substitution	AGC-GGC	703	Ser-Gly	12.6	1049
P6 <sup>a,b</sup>	E	Substitution	ATG-ATC	752	Met-Ile	5.4	1960
P7 <sup>a,b</sup>	E	Substitution	TAT-TGT	761	Tyr-Cys	2.2	1850
P8 <sup>a,b</sup>	E	Substitution	TAT-TGT	761	Tyr-Cys	3.0	1992
P9 <sup>b</sup>	E	Substitution	TAT-TGT	761	Tyr-Cys		
P10	F	Substitution	ATG-ATA	780	Met-Ile	6.5	500
P11	F	Substitution	CAG-GAG	798	Gln-Glu	1.4	1756
P12	G	Substitution	CGT-TGT	840	Arg-Cys	2.9	749
P13	G	Substitution	CGT-TGT	840	Arg-Cys		
P14	G	Substitution	CGC-CAC	855	Arg-His	4.3	587
P15	H	Substitution	ATT-ATG	869	Ile-Met	3.1	977

<sup>a</sup>The three cases that had two mutations each.<sup>b</sup>Denotes mutations not present in the AR database (11).**Relationship between Masculinization Score, AR Binding, and AR Gene Mutational Analysis**

AR binding studies, within the PAIS group, were performed in 10 cases with a masculinization score of less than 2, in 102 cases with a score between 2 and 6, and in 8 cases with a score above 6. There were no significant differences between the  $B_{max}$  or the  $K_d$  values for these three groups. Among the 57 mutation-positive cases of CAIS, AR binding was normal in 2 cases, 22 cases had zero binding, 11 cases had abnormal binding, and in 22 cases binding studies were not done (figure 14.3). In the group of 12 mutation-negative cases, 4 had zero binding, 3 had normal binding, 1 had abnormal binding, and in 4 cases binding studies were not performed.

In the PAIS group with identifiable mutations, there was one case with normal binding and 11 with abnormal binding. In the group of mutation-negative cases, 14 cases had abnormal binding, 3 cases had zero binding, 12 cases had normal binding, and in 2 cases binding studies were not performed. For the PAIS group with abnormal binding, the median  $B_{max}$  of the mutation-positive cases at  $1803 \times 10^{-18}$  moles/ $\mu$ g DNA (range, 457–2057) was higher than that of the mutation-negative cases at  $821 \times 10^{-18}$  moles/ $\mu$ g DNA (range, 83–1780) ( $P = 0.01$ , Wilcoxon signed rank test). In addition, in the mutation-positive cases with abnormal binding a median  $K_d$  of  $3.0 \times 10^{-10}$  M (range, 1.4–12.6) was higher than the median  $K_d$  value of  $1.4 \times 10^{-10}$  M for the mutation-negative cases (range, 0.6–11.2) ( $P = 0.003$ , WSR).



**Figure 14.2**

Distribution and nature of mutations over the eight exons (A-H) of the AR gene. The type of defect in androgen binding in these cases of CAIS and PAIS is also shown. There was one additional gene defect, a complete deletion of the AR gene with zero binding, which is not included in the figure.

## Discussion

Morris originally reported a series of eighty-two individuals with AIS (17). Since then, endocrine and molecular studies of this syndrome have provided useful insights into the function of the AR. Owing to the rarity of the condition, the literature does not contain a sizeable series of cases where clinical, as well as molecular, data are adequately collated. Against a background of clinical and biochemical evaluation of suspected cases of AIS, this study reports the results of androgen-binding assays and mutational analyses of the AR gene in the majority of cases as performed in a single referral center. This unique study, made possible only because of the ready cooperation of numerous clinicians, has allowed the creation of a large database consisting of clinical, biochemical and molecular genetic information about AIS, as well as other conditions associated with ambiguous genitalia. In any such multicenter study, it is inevitable that some data will be incomplete. There was a bias toward reporting younger age cases, thus explaining the relative lack of cases presenting with primary amenorrhea. Nevertheless, there is sufficient information from this study to draw conclusions about a number of aspects of the pathophysiology of AIS.

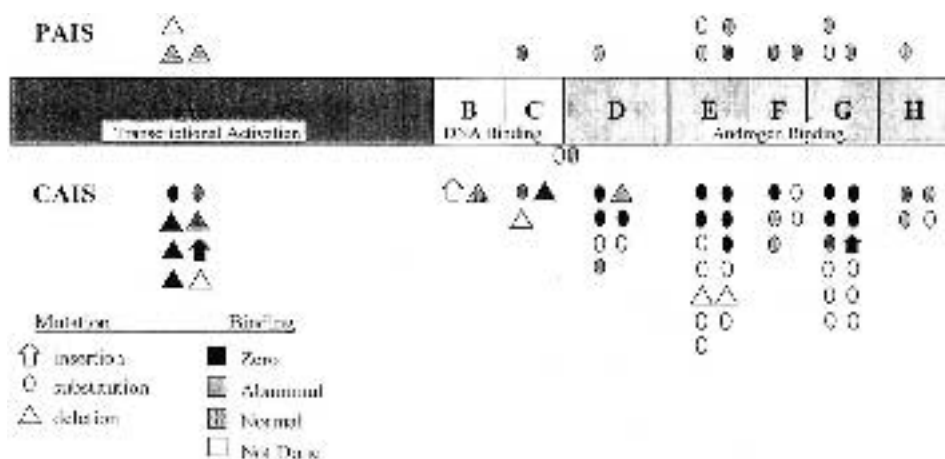
Presentation in CAIS in this study was predominantly by the discovery of a hernia and emphasizes the importance of considering AIS in any female infant with inguinal hernia. Estimates of the incidence of AIS in such infants have ranged from 1–12%, suggesting that any girl with an inguinal hernia should have a kar-

yotype performed (18, 19). Whereas nearly half (34 of 75 cases) of the CAIS cases had a family history of AIS, only 22% presented with a positive family history, indicating that more families with affected individuals need genetic counseling.

The diagnosis of AIS, particularly PAIS, includes confirmation of adequate testosterone biosynthesis and metabolism. However, in this study testosterone measurements and results of HCG stimulation tests were not available in a number of cases of male under-masculinization. Urinary steroid excretion as measured by chromatographic analysis was performed in less than one third of the cases of PAIS. It is, therefore, possible that some cases of male under-masculinization labeled as PAIS are not due to androgen insensitivity. A lower incidence of a positive family history in the PAIS group is further evidence for greater etiological heterogeneity in this cohort, as well as suggesting that PAIS can arise sporadically. In our study, there were 13 *clinically diagnosed* cases of CAIS in whom no AR mutation or AR-binding abnormalities were detected, but 3 of these cases had an X-linked family history of male under-masculinization; in an additional 6 cases, appropriate investigations had been performed to exclude causes other than AIS. In the PAIS cohort, there were 78 such cases; 9 had a positive X-linked family history and an additional 59 cases had been adequately investigated according to our opinion (20).

It is unclear whether testicular tumors are more common in AIS patients as compared with those who have simple cryptorchidism in whom the prevalence of the premalignant state of carcinoma in situ has been reported to be as high as 3% (21). Histology of the testes was not available in a number of cases in this study, and numerous pathologists reported the histology in the remainder. No instances of neoplasm were reported, but there is a need to perform a more detailed study of premalignant markers in these cases (22, 23). The hamartomatous nodule has been described before in AIS; its prevalence in testes of unaffected individuals is unknown, and the etiology remains speculative (24). Leydig cell hyperplasia is often seen in CAIS, and it is possible that the hamartoma may also be due to LH hyperstimulation. Most AIS cases in this study had gonadectomy performed before puberty. The bias toward a younger population may have influenced the results as the likelihood of encountering neoplasia is higher from the 3rd decade of life onward (25).

The preference for early gonadectomy was clear in this study; girls with CAIS who had postpubertal gonadectomy were older at the time of presentation. The case for early gonadectomy rests mainly on the reported increased occurrence of atypical germ cells described as carcinoma in situ (26) or intratubular germ cell neoplasia (27) in prepubertal cases of AIS,



**Figure 14.3**

Distribution of masculinization scores according to sex of rearing in PAIS. Cases in the *black* area were reared as boys, in the *white* area as girls, and in the *grey* area as girls or boys.

as well as extrapolated data from cases of gonadal dysgenesis where overt testicular neoplasia can occur before puberty. However, the youngest reported case of AIS with an overt germ cell tumor was 14-yr-old at presentation (28). Testicular germ cell tumors generally have an excellent prognosis and may be detected early with the help of routine ultrasound imaging and tumor markers (29).

Other arguments for early gonadectomy include the perception among doctors, as well as parents, that the affected girl will suffer less distress if she is not involved in the practical issues surrounding gonadectomy. Data to support this argument are, however, lacking. It is also unknown whether sustained exposure to aromatized derivatives of testosterone has any significant effect on higher CNS centers involved in the development of sexual identity (30). Furthermore, a recent study of six adults with CAIS suggested that early oestrogen replacement combined with gonadectomy in late puberty may be beneficial for bone mineralization (31). It is possible that a later onset of estrogen replacement in children with early gonadectomy is detrimental for bone mineralization in the long term. Allowing spontaneous development of puberty may benefit self-esteem during adolescence with gonadectomy then performed in a climate of informed consent and full discussion. An opinion survey of pediatric and adult endocrinologists and gynecologists on this issue showed no evidence of unanimity (personal observations).

The definitive diagnosis of AIS is based both on clinical examination and the results of appropriate investigations. A number of cases reported as AIS were not as comprehensively investigated as recommended by current protocols for evaluation of male under-masculinization (32–34). Standardized diagnostic approach to male under-masculinization as proposed

by Albers et al. (34) has clarity but should also emphasize the importance of investigating in an expeditious and cost-effective manner that is appropriate to local circumstances.

Our recent studies of masculinization scores in all cases of male under-masculinization showed similar results to that observed with this subset of AIS patients (35). Whereas the median scores were different for the two sexes, there was a substantial overlap. The Prader-type classification attempts to fit every case of ambiguous genitalia into one of six types (36), whereas the masculinization score, in contrast, independently considers each physical feature. The score, itself, cannot influence decisions about sex assignment, but could be used to decide when to investigate or to seek a specialist opinion. In this study, no case was raised female if the masculinization score was more than 9. Consequently, it would seem reasonable to seek a specialist opinion in cases of under-masculinized male newborns with a score of 10 or less. Such guidelines need testing prospectively before their application in clinical practice, but go some way toward meeting a recent demand for some guidelines as when to investigate further for abnormal genitalia (33). An added benefit of the masculinization score was the facility to objectively compare biochemical, genetic, and clinical features of these AIS cases.

AR-binding assays using genital skin fibroblasts have provided useful information about the pathophysiology of AIS (4–8). However, they are laborious and there is marked variability in AR-binding capacity in normal individuals, as reflected in the normal reference range. The site of skin biopsy and tissue culture conditions contributes to the variation in binding characteristics. Nevertheless, some notable differences in AR binding were observed for the two forms of AIS.

Binding was more likely to be abnormal in the complete form of AIS; a number of PAIS cases had abnormal binding, but rarely as severe (zero binding) as that seen in CAIS. Our data would suggest that chances of finding a mutation were much higher in CAIS and results of androgenbinding studies may not influence the decision to screen the AR gene. However, this may not apply to PAIS cases where an altered  $K_d$  may be a useful pointer to an AR gene mutation.

Mutations in CAIS occurred throughout the coding region of the AR gene, but mainly affected the ligand-binding domain and particularly involved exon E. This distribution of mutations is consistent with that reported in recent reviews (1, 37). Mutations affecting the DNA-binding domain, encoded by exons B and C, have previously been described in PAIS (11, 37), but there are only two cases of PAIS where mutations have been described in exon A, encoding for the amino terminal transcriptional activation domain (38, 39). The mutations found in our series of PAIS cases included a shortening of the polyglutamine repeat in exon A in three cases who were related to each other and had previously been reported by McPhaul et al. (40). The average length of this CAG repeat region has been reported at  $21 \pm 2$  repeats and can be as low as 11 in a normal, mixed-sex population (41, 42). The shortened repeat sequence coexisted with another mutation in exon E (table 14.3). Transfection studies performed by McPhaul et al. (40) showed that this shortened repeat sequence did not affect AR binding as much as the exon E mutation (40). The shortened repeat sequence was the lowest we have observed in our cohort. It was included in the list of mutations in table 14.3 because we believe that such a marked degree of shortening of the CAG repeats in AR cannot be ignored in the context of AIS. A case of CAIS caused by a mutation affecting the DNA-binding domain was, as anticipated, associated with normal AR binding. However, there were cases with abnormal binding who did not have a mutation in the coding region of the AR gene. Studies of AR transcription (43) and the activity of transfected androgen-responsive reporter genes in genital skin fibroblast cultures (44) may elucidate the underlying defect in androgen action.

Our experience would confirm the use of SSCA as a highly sensitive screening technique because no genetic abnormalities were discovered by direct sequencing in those cases where SSCA was normal. This study did not demonstrate a relationship between either the masculinization score and the results of androgen-binding studies or the nature of AR gene mutations. Cases of CAIS had been selected for mutational analysis because the initial biochemical evaluation had excluded other causes of male under-masculinization and the phenotype was unambiguously female. With

such a high yield of mutations, it can be argued that androgen-binding studies are not needed for the CAIS patient. However, a genital skin biopsy for fibroblast culture may also serve as an essential source of AR messenger RNA to screen for mutations in noncoding regions of the AR gene (45, 46). An altered receptor-binding affinity in mutation-positive cases of PAIS in comparison with mutation-negative cases illustrates the value of binding studies in selecting PAIS cases for mutation screening. In this series, only one mutation-positive case of PAIS had normal binding; mutation analysis had been performed because of a positive family history and biochemical evaluation had excluded other causes of male under-masculinization. In the absence of a genital skin fibroblast line, direct mutation analysis is the only option but should be undertaken when comprehensive clinical and biochemical evaluation has excluded other causes of male under-masculinization. Our database contains numerous examples of isolated cases of male under-masculinization whose clinical and biochemical features are consistent with PAIS but in whom androgen studies are normal. A certain number can be screened for AR gene mutations, but a positive yield is likely to be low. It remains a challenge to determine the nature of a congenital urogenital disorder that seems to be associated with normal androgen production and metabolism.

This study of a large series of patients with male under-masculinization indicates that the clinical diagnosis of AIS can be confirmed in a significant number of cases by a combination of androgen-binding studies and mutational analysis. There is some correlation between the phenotypic features and the type of AR gene mutation. Phenotypic heterogeneity among clinically diagnosed cases of AIS emphasizes the need for appropriate comprehensive evaluation of male under-masculinization. It is clear that although PAIS remains a common diagnosis in cases of male under-masculinization, specific mutations involving the X-linked AR gene account for only a proportion of the cases.

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The 47,XXY genotype occurs in 1 in 1,000 conceptions but, due to spontaneous abortion, is seen in only 1 in 2,000 live births (1). Individuals with a 47,XXY karyotype are generally males, presenting with the clinical phenotype of Klinefelter syndrome (2). A few 47,XXY females with testicular feminization have been described (3–9). Five of these rare cases were diagnosed as having androgen insensitivity (testicular feminization) syndrome resulting from mutations in the androgen receptor gene (3–6, 9).

Male development is triggered by transient expression of the SRY gene, which initiates a cascade of gene interactions ultimately leading to the formation of a testis from the indifferent fetal gonad. Mutations in this gene have been implicated in gonadal dysgenesis (female 46,XY) (10, 11). However, the gene has been little studied in 47,XXY females (7, 8).

### Case Report

A 34-year-old woman presented with complete testicular feminization. Physical examination revealed a healthy woman with normal intelligence; her height and weight were 180 cm and 88 kg, respectively.

The secondary sexual characteristics were small and tubular breasts (Tanner 3) and scarce pubic hair. Pelvic examination revealed hypoplasia of the labial folds, clitoris and vagina. Pelvic ultrasound showed absence of the uterus, ovaries and wolffian and müllerian ducts, and laparoscopy confirmed the absence of these organs.

Laboratory investigation disclosed values for follicle-stimulating hormone of 36.24 mIU/mL, luteinizing hormone of 10.31 mIU/mL, testosterone hormone of 0.32 ng/mL and estradiol of 17.70 pg/mL.

The family history revealed no consanguinity, miscarriages or ambiguous genitalia in females.

Lymphocyte cultures for chromosomal analysis with GTC (G-bands by trypsin using Giemsa stain) (figure 15.1A) and CBG (G-bands using barium hydroxide and Giemsa stain) (12) (figure 15.1B) showed a 47,XXY karyotype.

The fluorescence in situ hybridization (FISH) procedure was done using a biotin-labeled DNA probe

containing complementary centromeric  $\alpha$  satellite sequences of chromosome X (DXZ1) and an avidin-labeled DNA probe with complementary centromeric  $\alpha$  satellite sequences of chromosome Y (DYZ1). The DXZ1 was detected with rodamine-labeled avidin and DYZ1 with FITC-labeled biotin using phenylendiamine dihydrochloride as contrast dye (13). A total of 500 cells (50 metaphase and 450 interphase) were examined on a standard epifluorescence-equipped microscope (Axiophot, Carl Zeiss, Oberkochen, Germany). FISH showed normal chromosomal structures (figure 15.1C), and no evidence of mosaicism.

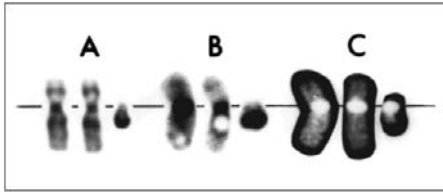
Polymerase chain reaction using the primers SRY1 (5'-ATAAGTATCGACCTCGTCGGAAG-3') and SRY2 (5'-GCACTTCGCTGCAGAGTACCGAAG-3') were a positive for the SRY gene.

### Discussion

Cytogenetic and molecular analyses showed that the genotype of this phenotypically female patient was 47,XXY and that the X and Y chromosomes were structurally unaltered, with a normal SRY gene. These results are similar to those in the 2 patients previously reported (7, 8).

Röttger et al. (14) reported a fertile woman with a 47,XXY karyotype carrying a structurally altered Y chromosome and negative SRY gene. As a consequence, Yp material, including SRY, was replaced by the terminal sequences of the PRKX gene. Fertility was attributed to the presence of the additional X chromosome, which is missing from XY gonadal dysgenesis females.

There are 2 possible origins of 47,XXY in patients with testicular feminization. First, maternal nondisjunction during meiosis I results in 1 X chromosome carrying a mutation (heterozygosity) of the androgen receptor. The expression of the disorder is explained by the random nature of X inactivation in XXY patients because, overall, a defect resulting from a mutant androgen receptor allele on 1 X chromosome is masked by the effect of the normal allele on the other X chromosome. Second, maternal nondisjunction



**Figure 15.1**  
An XXY chromosome from the patient. (A) GTG banded. (B) CBG banded. (C) FISH.

during meiosis II results in 2 X chromosomes carrying a presumptive mutation (homozygosity) (9).

Current data on X chromosome gain in these patients suggest a potential oncogenic role in testicular germ cell tumors (15).

In conclusion, this is the third report on a case of 47,XXY with a female phenotype, normal Y chromosome and positive SRY. This suggests that the phenotypic sex in these patients might be due to the involvement of other sex-determining genes, such as androgen receptor, Wilms tumor gene 1, EMX2, XH-2, GATA-4, LIM1, steroidogenic factor 1, SRY box-related gene 9, WNT-4 and DAX-1.

For patients with testicular feminization we recommend molecular analysis to expand knowledge of the disease and develop treatments that are useful clinically.

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In 1843 Levi Suydam, a twenty-three-year-old resident of Salisbury, Connecticut, asked the town board of selectmen to validate his right to vote as a Whig in a hotly contested local election. The request raised a flurry of objections from the opposition party, for reasons that must be rare in the annals of American democracy: it was said that Suydam was more female than male and thus (some eighty years before suffrage was extended to women) could not be allowed to cast a ballot. To settle the dispute a physician, one William James Barry, was brought in to examine Suydam. And, presumably upon encountering a phallus, the good doctor declared the prospective voter male. With Suydam safely in their column the Whigs won the election by a majority of one.

Barry's diagnosis, however, turned out to be somewhat premature. Within a few days he discovered that, phallus notwithstanding, Suydam menstruated regularly and had a vaginal opening. Both his/her physique and his/her mental predispositions were more complex than was first suspected. S/he had narrow shoulders and broad hips and felt occasional sexual yearnings for women. Suydam's "feminine propensities, such as a fondness for gay colors, for pieces of calico, comparing and placing them together, and an aversion for bodily labor, and an inability to perform the same, were remarked by many," Barry later wrote. It is not clear whether Suydam lost or retained the vote, or whether the election results were reversed.

Western culture is deeply committed to the idea that there are only two sexes. Even language refuses other possibilities: thus to write about Levi Suydam I have had to invent conventions—s/he and his/her—to denote someone who is clearly neither male nor female or who is perhaps both sexes at once. Legally, too, every adult is either man or woman, and the difference, of course, is not trivial. For Suydam it meant the franchise; today it means being available for, or exempt from, draft registration, as well as being subject, in various ways, to a number of laws governing marriage, the family and human intimacy. In many parts of the United States, for instance, two people legally regis-

tered as men cannot have sexual relations without violating anti-sodomy statutes.

But if the state and the legal system have an interest in maintaining a two-party sexual system, they are in defiance of nature. For biologically speaking, there are many gradations running from female to male; and depending on how one calls the shots, one can argue that along that spectrum lie at least five sexes—and perhaps even more.

For some time medical investigators have recognized the concept of the intersexual body. But the standard medical literature uses the term intersex as a catch-all for three major subgroups with some mixture of male and female characteristics: the so-called true hermaphrodites, whom I call herms, who possess one testis and one ovary (the sperm- and egg-producing vessels, or gonads); the male pseudohermaphrodites (the "merms"), who have testes and some aspects of the female genitalia but no ovaries; and the female pseudohermaphrodites (the "merms"), who have ovaries and some aspects of the male genitalia but lack testes. Each of those categories is in itself complex; the percentage of male and female characteristics, for instance, can vary enormously among members of the same subgroup. Moreover, the inner lives of the people in each subgroup—their special needs and their problems, attractions and repulsions—have gone unexplored by science. But on the basis of what is known about them I suggest that the three intersexes, herm, merm and ferm, deserve to be considered additional sexes each in its own right. Indeed, I would argue further that sex is a vast, infinitely malleable continuum that defies the constraints of even five categories.

Not surprisingly, it is extremely difficult to estimate the frequency of intersexuality, much less the frequency of each of the three additional sexes: it is not the sort of information one volunteers on a job application. The psychologist John Money of Johns Hopkins University, a specialist in the study of congenital sexual-organ defects, suggests intersexuals may constitute as many as 4 percent of births. As I point out to my students at Brown University, in a student body of about 6,000

that fraction, if correct, implies there may be as many as 240 intersexuals on campus—surely enough to form a minority caucus of some kind.

In reality though, few such students would make it as far as Brown in sexually diverse form. Recent advances in physiology and surgical technology now enable physicians to catch most intersexuals at the moment of birth. Almost at once such infants are entered into a program of hormonal and surgical management so that they can slip quietly into society as “normal” heterosexual males or females. I emphasize that the motive is in no way conspiratorial. The aims of the policy are genuinely humanitarian, reflecting the wish that people be able to “fit in” both physically and psychologically. In the medical community, however, the assumptions behind that wish—that there be only two sexes, that heterosexuality alone is normal, that there is one true model of psychological health—have gone virtually unexamined.

The word hermaphrodite comes from the Greek names Hermes, variously known as the messenger of the gods, the patron of music, the controller of dreams or the protector of livestock, and Aphrodite, the goddess of sexual love and beauty. According to Greek mythology, those two gods parented Hermaphroditus, who at age fifteen became half male and half female when his body fused with the body of a nymph he fell in love with. In some true hermaphrodites the testis and the ovary grow separately but bilaterally; in others they grow together within the same organ, forming an ovo-testis. Not infrequently, at least one of the gonads functions quite well, producing either sperm cells or eggs, as well as functional levels of the sex hormones—androgens or estrogens. Although in theory it might be possible for a true hermaphrodite to become to a child, in practice the appropriate ducts and tubes are not configured so that egg and sperm can meet.

In contrast with the true hermaphrodites, the pseudohermaphrodites possess two gonads of the same kind along with the usual male (XY) or female (XX) chromosomal makeup. But their external genitalia and secondary sex characteristics do not match their chromosomes. Thus merms have testes and XY chromosomes, yet they also have a vagina and a clitoris, and at puberty they often develop breasts. They do not menstruate, however. Fems have ovaries, two X chromosomes and sometimes a uterus, but they also have at least partly masculine external genitalia. Without medical intervention they can develop beards, deep voices and adult-size penises.

No classification scheme could more than suggest the variety of sexual anatomy encountered in clinical practice. In 1969, for example, two French investigators, Paul Guinet of the Endocrine Clinic in Lyons and

Jacques Decourt of the Endocrine Clinic in Paris, described ninety-eight cases of true hermaphroditism—again, signifying people with both ovarian and testicular tissue—solely according to the appearance of the external genitalia and the accompanying ducts. In some cases the people exhibited strongly feminine development. They had separate openings for the vagina and the urethra, a cleft vulva defined by both the large and the small labia, or vaginal lips, and at puberty they developed breasts and usually began to menstruate. It was the oversize and sexually alert clitoris, which threatened sometimes at puberty to grow into a penis, that usually impelled them to seek medical attention. Members of another group also had breasts and a feminine body type, and they menstruated. But their labia were at least partly fused, forming an incomplete scrotum. The phallus (here an embryological term for a structure that during usual development goes on to form either a clitoris or a penis) urinated through a urethra that opened into or near the vagina.

By far the most frequent form of true hermaphrodite encountered by Guinet and DeCourt—55 percent—appeared to have a more masculine physique. In such people the urethra runs either through or near the phallus, which looks more like a penis than a clitoris. Any menstrual blood exits periodically during urination. But in spite of the relatively male appearance of the genitalia, breasts appear at puberty. It is possible that a sample larger than ninety-eight so-called true hermaphrodites would yield even more contrasts and subtleties. Suffice it to say that the varieties are so diverse that it is possible to know which parts are present and what is attached to what only after exploratory surgery.

The embryological origins of human hermaphrodites clearly fit what is known about male and female sexual development. The embryonic gonad generally chooses early in development to follow either a male or a female sexual pathway; for the ovo-testis, however, that choice is fudged. Similarly, the embryonic phallus most often ends up as a clitoris or a penis, but the existence of intermediate states comes as no surprise to the embryologist. There are also uro-genital swellings in the embryo that usually either stay open and become the vaginal labia or fuse and become a scrotum. In some hermaphrodites, though, the choice of opening or closing is ambivalent. Finally, all mammalian embryos have structures that can become the female uterus and the fallopian tubes, as well as structures that can become part of the male sperm-transport system. Typically either the male or the female set of those primordial genital organs degenerates, and the remaining structures achieve their sex-appropriate future. In hermaphrodites both sets of organs develop to varying degrees.

Intersexuality itself is old news. Hermaphrodites, for instance, are often featured in stories about human origins. Early biblical scholars believed Adam began life as a hermaphrodite and later divided into two people—a male and a female—after falling from grace. According to Plato there once were three sexes—male, female and hermaphrodite—but the third sex was lost with time.

Both the Talmud and the Tosefta, the Jewish books of law, list extensive regulations for people of mixed sex. The Tosefta expressly forbids hermaphrodites to inherit their fathers' estates (like daughters), to seclude themselves with women (like sons) or to shave (like men). When hermaphrodites menstruate they must be isolated from men (like women); they are disqualified from serving as witnesses or as priests (like women), but the laws of pederasty apply to them.

In Europe a pattern emerged by the end of the Middle Ages that, in a sense, has lasted to the present day: hermaphrodites were compelled to choose an established gender role and stick with it. The penalty for transgression was often death. Thus in the 1600s a Scottish hermaphrodite living as a woman was buried alive after impregnating his/her master's daughter.

For questions of inheritance, legitimacy, paternity, succession to title and eligibility for certain professions to be determined, modern Anglo-Saxon legal systems require that newborns be registered as either male or female. In the U.S. today sex determination is governed by state laws. Illinois permits adults to change the sex recorded on their birth certificates should a physician attest to having performed the appropriate surgery. The New York Academy of Medicine, on the other hand, has taken an opposite view. In spite of surgical alterations of the external genitalia, the academy argued in 1966, the chromosomal sex remains the same. By that measure, a person's wish to conceal his or her original sex cannot outweigh the public interest in protection against fraud.

During this century the medical community has completed what the legal world began—the complete erasure of any form of embodied sex that does not conform to a male-female, heterosexual pattern. Ironically, a more sophisticated knowledge of the complexity of sexual systems has led to the repression of such intricacy.

In 1937 the urologist Hugh H. Young of Johns Hopkins University published a volume titled *Genital Abnormalities, Hermaphroditism and Related Adrenal Diseases*. The book is remarkable for its erudition, scientific insight and open-mindedness. In it Young drew together a wealth of carefully documented case histories to demonstrate and study the medical treatment of such “accidents of birth.” Young did not pass judgment on the people he studied, nor did he attempt to

coerce into treatment those intersexuals who rejected that option. And he showed unusual even-handedness in referring to those people who had had sexual experiences as both men and women as “practicing hermaphrodites.”

One of Young's more interesting cases was a hermaphrodite named Emma who had grown up as a female. Emma had both a penis-size clitoris and a vagina, which made it possible for him/her to have “normal” heterosexual sex with both men and women. As a teenager Emma had had sex with a number of girls to whom s/he was deeply attracted; but at the age of nineteen s/he had married a man. Unfortunately, he had given Emma little sexual pleasure (though he had had no complaints), and so throughout that marriage and subsequent ones Emma had kept girlfriends on the side. With some frequency s/he had pleasurable sex with them. Young describes his subject as appearing “to be quite content and even happy.” In conversation Emma occasionally told him of his/her wish to be a man, a circumstance Young said would be relatively easy to bring about. But Emma's reply strikes a heroic blow for self-interest:

Would you have to remove that vagina? I don't know about that because that's my meal ticket. If you did that, I would have to quit my husband and go to work, so I think I'll keep it and stay as I am. My husband supports me well, and even though I don't have any sexual pleasure with him, I do have lots with my girlfriends.

Yet even as Young, was illuminating intersexuality with the light of scientific reason, he was beginning its suppression. For his book is also an extended treatise on the most modern surgical and hormonal methods of changing intersexuals into either males or females. Young may have differed from his successors in being less judgmental and controlling of the patients and their families, but he nonetheless supplied the foundation on which current intervention practices were built.

By 1969, when the English physicians Christopher J. Dewhurst and Ronald R. Gordon wrote *The Intersexual Disorders*, medical and surgical approaches to intersexuality had neared a state of rigid uniformity. It is hardly surprising that such a hardening of opinion took place in the era of the feminine mystique—of the post-Second World War flight to the suburbs and the strict division of family roles according to sex. That the medical consensus was not quite universal (or perhaps that it seemed poised to break apart again) can be gleaned from the near-hysterical tone of Dewhurst and Gordon's hook, which contrasts markedly with the calm reason of Young's founding work. Consider their opening description of an intersexual newborn:

One can only attempt to imagine the anguish of the parents. That a newborn should have a deformity...[affecting] so

fundamental an issue as the very sex of the child . . . is a tragic event which immediately conjures up visions of a hopeless psychological misfit doomed to live always as a sexual freak in loneliness and frustration.

Dewhurst and Gordon warned that such a miserable fate would, indeed, be a baby's lot should the case be improperly managed; "but fortunately," they wrote, "with correct management the outlook is infinitely better than the poor parents—emotionally stunned by the event—or indeed anyone without special knowledge could ever imagine."

Scientific dogma has held fast to the assumption that without medical care hermaphrodites are doomed to a life of misery. Yet there are few empirical studies to back up that assumption, and some of the same research gathered to build a case for medical treatment contradicts it. Francies Benton, another of Young's practicing hermaphrodites, "had not worried over his condition, did not wish to be changed, and was enjoying life." The same could be said of Emma, the opportunistic hausfrau. Even Dewhurst and Gordon, adamant about the psychological importance of treating intersexuals at the infant stage, acknowledged great success in "changing the sex" of older patients. They reported on twenty cases of children reclassified into a different sex after the supposedly critical age of eighteen months. They asserted that all the reclassifications were "successful," and they wondered then whether reregistration could be "recommended more readily than [had] been suggested so far."

The treatment of intersexuality in this century provides a clear example of what the French historian Michel Foucault has called *biopower*. The knowledge developed in biochemistry, embryology, endocrinology, psychology and surgery has enabled physicians to control the very sex of the human body. The multiple contradictions in that kind of power call for some scrutiny. On the one hand, the medical "management" of intersexuality certainly developed as part of an attempt to free people from perceived psychological pain (though whether the pain was the patient's, the parents' or the physician's is unclear). And if one accepts the assumption that in a sex-divided culture people can realize their greatest potential for happiness and productivity only if they are sure they belong to one of only two acknowledged sexes, modern medicine has been extremely successful.

On the other hand, the same medical accomplishments can be read not as progress but as a mode of discipline. Hermaphrodites have unruly bodies. They do not fall naturally into a binary classification; only a surgical shoehorn can put them there. But why should we care if a "woman," defined as one who has breasts, a vagina, a uterus and ovaries and who menstruates, also has a clitoris large enough to penetrate the vagina

of another woman? Why should we care if there are people whose biological equipment enables them to have sex "naturally" with both men and women? The answers seem to lie in a cultural need to maintain clear distinctions between the sexes. Society mandates the control of intersexual bodies because they blur and bridge the great divide. Inasmuch as hermaphrodites literally embody both sexes, they challenge traditional beliefs about sexual difference: they possess the irritating ability to live sometimes as one sex and sometimes the other, and they raise the specter of homosexuality.

But what things were altogether different? Imagine a world in which the same knowledge that has enabled medicine to intervene in the management of intersexual patients has been placed at the service of multiple sexualities. Imagine that the sexes have multiplied beyond currently imaginable limits. It would have to be a world of shared powers. Patient and physician, parent and child, male and female, heterosexual and homosexual—all those oppositions and others would have to be dissolved as sources of division. A new ethic of medical treatment would arise, one that would permit ambiguity in a culture that had overcome sexual division. The central mission of medical treatment would be to preserve life. Thus hermaphrodites would be concerned primarily not about whether they can conform to society but about whether they might develop potentially life-threatening conditions—hernias, gonadal tumors, salt imbalance caused by adrenal malfunction—that sometimes accompany hermaphroditic development. In my ideal world medical intervention for intersexuals would take place only rarely before the age of reason; subsequent treatment would be a cooperative venture between physician, patient and other advisers trained in issues of gender multiplicity.

I do not pretend that the transition to my utopia would be smooth. Sex, even the supposedly "normal," heterosexual kind, continues to cause untold anxieties in Western society. And certainly a culture that has yet to come to grips—religiously and, in some states, legally—with the ancient and relatively uncomplicated reality, of homosexual love will not readily embrace intersexuality. No doubt the most troublesome arena by far would be the rearing of children. Parents, at least since the Victorian era, have fretted, sometimes to the point of outright denial, over the fact that their children are sexual beings.

All that and more amply explains why intersexual children are generally squeezed into one of the two prevailing sexual categories. But what would be the psychological consequences of taking the alternative road—raising children as unabashed intersexuals? On the surface that task seems fraught with peril. What, for example, would happen to the intersexual child

amid the unrelenting cruelty of the school yard? When the time came to shower in gym class, what horrors and humiliations would await the intersexual as his/her anatomy was displayed in all its non-traditional glory? In whose gym class would s/he register to begin with? What bathroom would s/he use? And how on earth would Mom and Dad help shepherd him/her through the mine field of puberty?

In the past thirty years those questions have been ignored, as the scientific community has, with remarkable unanimity, avoided contemplating the alternative route of unimpeded intersexuality. But modern investigators tend to overlook a substantial body of case histories, most of them compiled between 1930 and 1960, before surgical intervention became rampant. Almost without exception, those reports describe children who grew up knowing they were intersexual (though they did not advertise it) and adjusted to their unusual status. Some of the studies are richly detailed—described at the level of gym-class showering (which most intersexuals avoided without incident); in any event, there is not a psychotic or a suicide in the lot.

Still, the nuances of socialization among intersexuals cry out for more sophisticated analysis. Clearly, before my vision of sexual multiplicity can be realized, the first openly intersexual children and their parents will have to be brave pioneers who will bear the brunt of society's growing pains. But in the long view—though it could take generations to achieve—the prize might be a society in which sexuality is something to be celebrated for its subtleties and not something to be teased or ridiculed.



## Introduction

In the fifteen years since estrogen biosynthesis was first shown to occur in human fetal brain tissue (1), aromatase activity has been identified in the central nervous systems of a wide variety of vertebrate species (2–4). In mammals, this activity has been demonstrated to be crucial for the actions of androgens on reproductively-oriented neuroendocrine functions and behaviors. Thus, blockade of aromatase during early life interferes with androgen-induced sexual differentiation of the hypothalamic mechanisms controlling reproductive function, while in adulthood it impairs or abolishes the expression of androgen-dependent courtship and sexual behavior patterns. (For reviews, see references 5 and 6.) The role of aromatase in the sexual differentiation of non-reproductive functions of the central nervous system, however, remains ill-defined. In particular, although sex differences have been repeatedly observed in patterns of cerebral development and cognitive function, in man as well as experimental animals, virtually nothing is known of how these sex differences arise. This chapter reviews recent evidence suggesting that sex differences in cognitive function also may be the result of androgen action during early life, involving the cortex and hippocampus and mediated at least in part through local estrogen biosynthesis.

## Distribution of Aromatase Activity in the Developing Mammalian Brain

In adult mammals, aromatase is concentrated in the hypothalamus, the preoptic area, and the cortical and medial nuclei of the amygdala (7–9). This distribution pattern corresponds well with sites of androgen sensitivity in the brain. Defeminization of gonadotrophin release and sexual behavior can be induced by implants of testosterone into the developing hypothalamus (10); activation of masculine sexual behavior later in life can likewise be accomplished by local hypothalamic androgen implants (11); and morphological and biochemical responses to androgen have been demonstrated in areas

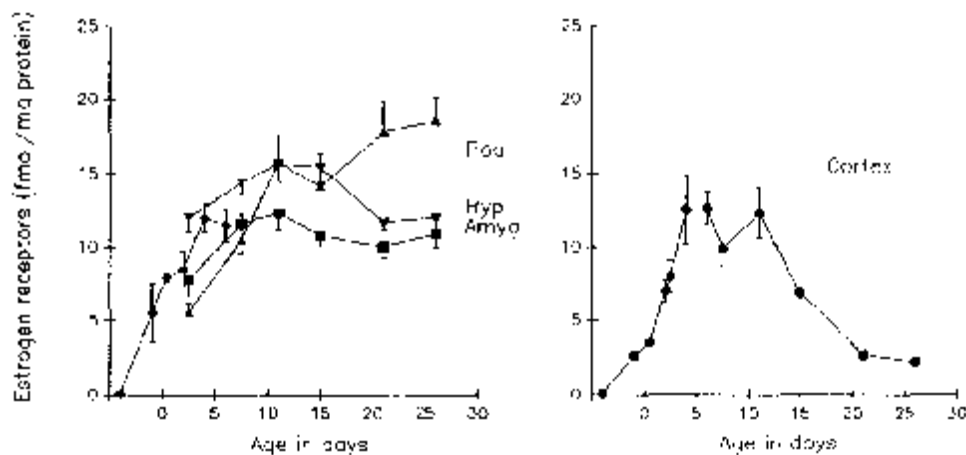
of the brain that contain high levels of aromatase activity (12–16).

The distribution of aromatase activity in the developing mammalian brain appears to be essentially similar to that in adulthood (17–19), although, as will be further discussed below, there may be subtle ontogenetic changes in the distribution of the enzyme. Aromatase appears in the brain at a fairly early stage of development. In rabbits, aromatase activity first becomes detectable in the diencephalon at around the 16th day of gestation. Thereafter, the activity of the enzyme increases sharply to a peak 3 days later, stabilizes between days 19 and 25, then rapidly declines (20). Fairly similar developmental profiles have been reported for the rat (17, 18, 21) and the ferret (19).

## Sex Differences in Cognitive Function: The Possible Role of Gonadal Steroids

The existence of sex differences in non-reproductive behaviors and cognitive function has been known for many years. In man, sex differences have been reported in play behavior, cognitive ability (in particular, visuospatial and language-related skills), cerebral lateralization and the occurrence of disorders such as dyslexia and epilepsy during childhood (22–25). These differences have been suggested to at least partially reflect sex differences in the regional development of the cerebral hemispheres (26, 27). In experimental animals, a large number of non-reproductive behaviors have been reported to be sexually differentiated (28). Interestingly, these differences include parameters that may be analogous to sexually/dimorphic functions in man—for example, play behavior (29, 30) and responses to cerebral cortical lesions (31–33).

These observations suggest that the development of the cerebral cortex may be sexually differentiated. Studies on maze-learning in rodents (34, 35) suggest that there may also be contributions to the sexual differentiation of cognitive ability from alterations in hippocampal function. This view is supported by a number of recent reports of sex differences in hippocampal



**Figure 17.1**

Development of estrogen receptors measured in cytosol fractions from cerebral cortex, hypothalamus, preoptic area, and amygdala of female rats during perinatal and early postnatal life. Between days  $-4$  and  $+6$  (day of birth = day 0), assays were performed on the pooled hypothalamus (Hyp), preoptic area (Poa), and amygdala (Amyg) (◆). In a separate series of experiments between days 3 and 25, these brain regions were analyzed individually. (Redrawn from Refs. 47 and 48, with permission.)

and cerebral cortical morphology. In rodents, significant differences have been demonstrated between males and females in hippocampal dendritic branching patterns (36) and in axonal sprouting within the hippocampus after disruption of septo-hippocampal afferents (37, 38). These differences appear to be the result of testosterone secretion in the male. In rats, sex differences have been reported in the symmetry of the cerebral cortex. In normal males, several regions of the cortex develop asymmetrically, the cortical layers being thicker on the right side of the brain than on the left (39, 40). This difference is not observed in either females or males castrated on the day of birth (41). Interestingly, stress during the perinatal period, which also interferes with masculine differentiation of reproductive function (42, 43), abolishes the right-over-left difference normally observed in the cortex of the male (40). In man, sex differences have been reported in cerebral hemispheric asymmetry (44) and in the anatomy of the corpus callosum (45, 46).

The mechanisms responsible for these sexual dimorphisms remain unknown. A reasonable hypothesis, however, is that the differentiation of these structures may be brought about through aromatase-dependent mechanisms essentially similar to those operating in the diencephalon. Strong circumstantial evidence supporting a role for estrogen in the sexual differentiation of the cerebral cortex comes from studies of the distribution of estrogen receptors in the developing brain. In rats (47, 48), mice (49), and ferrets (50), estrogen receptors appear at high concentrations in the cerebral cortex during perinatal and early postnatal life. These receptors appear to be principally located in layers 5–6 of the cortex (51, 52) and are present only during a

restricted phase of early life; during the second postnatal week their concentrations rapidly decline, reaching adult levels by around postnatal day 20 (figure 17.1). More recent studies in newborn rhesus monkeys have also demonstrated the existence of estrogen receptors in several areas of the cerebral cortex (53). There is every reason to suppose that these receptors are functional. Biochemical studies in rodents have demonstrated that they bind natural and synthetic estrogens, forming nuclear-bound receptor complexes (48). In rats, estrogen administration during the first two weeks of life accelerates cortical maturation (54–56), while under *in vitro* conditions, estradiol stimulates the outgrowth of neurites from explant cultures of newborn mouse cingulate cortex in much the same way as it does from similar cultures of the hypothalamus and preoptic area (57). That estrogen action might be involved in the sexual differentiation of cognitive behavior is also suggested by similarities between the effects of estrogen exposure during development and aspects of normal masculinization. As previously mentioned, men exhibit a different pattern of cerebral lateralization than women, expressed in terms of the localization of cortical functions and a more strongly lateralized pattern of responses to visual or auditory test stimuli. Hines has reported that women exposed prenatally to the synthetic estrogen, diethylstilbestrol, are more lateralized for responses to auditory stimuli than their unexposed siblings (58). Very recently, Williams et al. (34) have reported that estradiol benzoate administered over the first 9 days of life masculinizes the performance of female rats in a radial maze task designed to test visuospatial memory. These observations are reminiscent of the defeminizing effects of

early estrogen exposure on reproductive function (59) and clearly suggest that conversion of testosterone to estrogen may play a role in the sexual differentiation of cortical function.

The idea that local estrogen biosynthesis might be involved in sexual differentiation of regions of the brain subserving non-reproductive as well as reproductive functions is attractive, in that it offers a unifying mechanism for the actions of androgens on the developing central nervous system. There is, however, one major problem with this theory. The key element in the actions of testosterone on the mechanisms governing reproductive function in laboratory rodents is the aromatase enzyme complex. Unfortunately, studies of the distribution of aromatase activity in several different mammalian species have failed to convincingly demonstrate estrogen biosynthesis in cerebral cortical tissue (17–19). Likewise, while studies in the human fetus have demonstrated aromatase activity in the hippocampus (2), in adult rodents biochemical (60) and autoradiographic (61) studies have provided no evidence for estrogen formation in this region of the brain. These negative results, however, are inconclusive since they establish only that aromatase activity in the hippocampus and cerebral cortex is not in the same range as that in the hypothalamus, preoptic area, and amygdala; they leave open the possibility that the former two structures might contain lower, but nevertheless physiologically important, levels of the enzyme. In fact, previous studies have suggested that low levels of aromatase might be present in the cerebral cortex during early development (18, 62, 63); but definitive proof of this activity remained lacking, primarily because of difficulties in obtaining sufficiently large quantities of the presumed estrogen products to permit their unequivocal chemical characterization. These observations provided the impetus for the studies summarized below, in which we have re-evaluated the distribution of aromatase in the brains of three representative mammalian species (rhesus monkey, rat, and mouse), during early life.

#### Estrogen Biosynthesis in the Cerebral Cortex of the Developing Rhesus Monkey: Regional and Temporal Variations in Aromatase Activity

To further explore the possible role of aromatase in the primate central nervous system, we have re-examined the distribution of aromatase in the brain of the developing rhesus monkey (*Macaca mulatta*). Initial experiments examined the conversion of [ $^3\text{H}$ ]-androstenedione to [ $^3\text{H}$ ]-labeled estrogens in homogenates of different regions of the brain, from animals sacrificed during late fetal and early postnatal life (53).

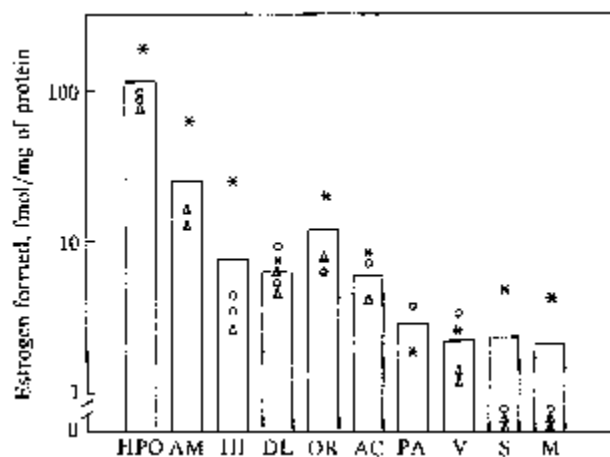


Figure 17.2

Aromatase activity in the brain of the neonatal rhesus monkey.  $\Delta$  Results from a 2-day-old male.  $\circ$  Results from a 5- and a 6-day-old female. \* Results from a female fetus (153 days postconception). Where the symbols for the male are shaded, this indicates that the tissue samples from the left and right hemispheres were assayed independently (shading of the left half of the symbol indicating results from the left hemispheric sample, and vice-versa). Histogram bars represent the mean values for each region, combining the data from all four animals. The means of the results from the two hemispheres in the 2-day-old male were used in calculating the overall mean estrogen formation rates. Results are plotted semilogarithmically. HPO—pooled hypothalamus and preoptic area; AM—amygdala; HI—hippocampus. Cortical regions: DL—dorsolateral prefrontal; OR—orbital prefrontal; AC—anterior cingulate; PA—parietal; V—Visual; S—somatosensory; M—motor. (Reprinted from Ref. 53 with permission.)

This time period was chosen on the basis of previous studies demonstrating that neonatal testosterone treatment masculinizes the behavioral response of infant rhesus monkeys to lesions in the orbital prefrontal cortex (32). After incubation with the radiolabeled substrate, metabolites were separated by phenolic partition and thin-layer chromatography, as previously described (18, 53). The identities of the estrogens recovered were confirmed by reverse isotope dilution and crystallization to constant specific activity.

Aromatase activity was detectable in almost all of the cerebral cortical, limbic, and diencephalic structures studied. Highest activities were observed in the hypothalamus/preoptic area and amygdala. However, surprisingly high aromatase activities were also observed in several regions of the association cortex (orbital prefrontal, dorsolateral prefrontal, and anterior cingulate), as well as in the hippocampus (figure 17.2). Lower, but still detectable, activities were observed in the parietal, visual, somatosensory, and motor cortex samples. Subsequent studies in our laboratories have confirmed and extended these findings (64, 65). Overall estrogen biosynthesis in the rhesus monkey brain declines rapidly during late fetal and early postnatal life. Fetal monkeys (127 and 135 days

postconception) exhibited measurable aromatase activities in all regions of the cerebral cortex. Postnatally, estrogen formation declines dramatically (>10-fold) in the primary cortical areas, but remains measurable in the hypothalamus/preoptic area and the orbital and dorsolateral regions of the prefrontal cortex (64, 65).

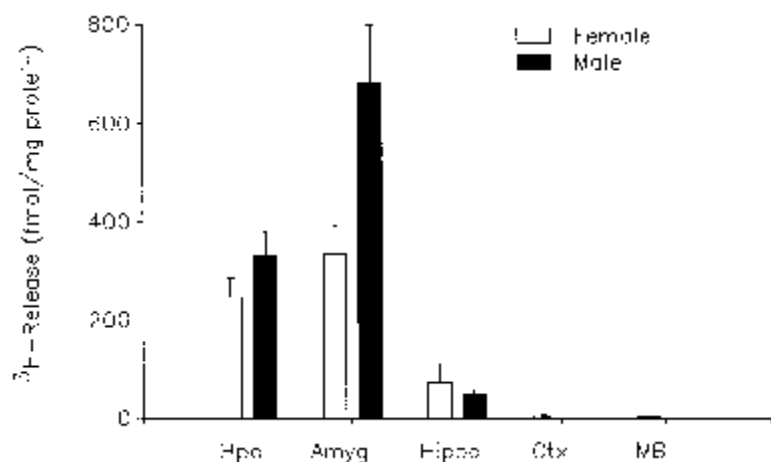
#### Distribution of Aromatase Activity in the Developing Rodent Brain

The presence of aromatase activity in the cortex of the developing monkey raised the possibility that the capacity for estrogen biosynthesis might also be more widespread in subprimate mammalian species than we had originally thought. The situation may, however, be complicated by species differences in the distribution of the aromatase enzyme: our preliminary data suggest that there are both similarities and differences between the patterns of cerebral estrogen biosynthesis in rodents and primates.

In collaboration with Dominique Toran-Allerand of Columbia University, we studied estrogen formation by different regions of the newborn mouse brain using a product isolation technique under in vitro organotypic culture conditions. Explants of different regions of the newborn mouse brain were grown for 3 days in Maximow tissue culture assemblies (66) in the presence of either [ $^3\text{H}$ ]androstenedione or 19-OH-[ $^3\text{H}$ ]androstenedione (8.7 pmol/culture). With the former isotope, the quantities of estrogen recovered were insufficient to allow unequivocal identification of the estrogen products. However, with 19-OH-[ $^3\text{H}$ ]androstenedione, which is a better substrate for estrogen biosynthesis than androstenedione (19, 21, 67), sufficient counts were recovered to allow characterization of the estrogens produced by reverse isotope

dilution and crystallization to constant specific activity. Highest rates of estrogen biosynthesis were observed in cultures from the hypothalamus and preoptic area (>250 fmol/ $\mu\text{g}$  tissue DNA). Aromatization was not detectable in cultures from the pons, medulla, cerebellum, or spinal cord. Low, but clearly measurable, levels of estrogen formation were observed in cultures from the hippocampus and anterior cingulate cortex—in the range of 10–20 fmol/ $\mu\text{g}$  DNA (68).

In the rat brain, we have attempted to identify and quantitate aromatase activity in tissue homogenates using both the product isolation methodology employed previously with monkeys (53) and a sensitive  $^3\text{H}$ -release assay modified from the procedure reported by Roselli et al. (69), using [ $1\beta$ - $^3\text{H}$ ]androstenedione as substrate. With the product isolation technique, we were unable to recover sufficient estrone or estradiol to allow positive identification by reverse isotopic dilution, except in the case of the hypothalamus, preoptic area and amygdala. Very low levels of radioactivity were recovered comigrating with the authentic estrogen standards from incubates with neonatal rat cerebral cortex. However, it was not possible to determine with any certainty that this radioactivity did in fact represent estrogen (18, 62). More recently, we have obtained essentially similar results with the tritium-release methodology. Figure 17.3 illustrates the distribution of aromatase activity in the brains of male and female rats, measured by this assay at postnatal day 5. High aromatase activities were observed in both sexes in the hypothalamus/preoptic area and amygdala. Somewhat lower activities were observed in the hippocampus. However, activities in the mid-brain and in samples of the frontal and anterior cingulate cortex were barely above the blank of the assay—at least 100-fold lower than those in the hypothalamus/preoptic area.



**Figure 17.3**

Distribution of aromatase activity in the brains of 5-day-old male and female rats. Results represent means [ $\pm$ SEM] of 4 observations in each case. HPO—hypothalamus + preoptic area; Amyg—amygdala; Hippo—hippocampus; Ctx—frontal and cingulate cortex; MB—midbrain.

### Implications of the Distribution of Aromatase Activity for Sexual Differentiation of Non-Reproductive Behaviors

The presence of aromatase activity in the cerebral cortex of the immature rhesus monkey suggests that local estrogen biosynthesis may play an important role in the developmental actions of androgens on this region of the brain. Although we have not systematically examined the prenatal development of aromatase in the monkey brain, studies from another laboratory suggest that the enzyme activity peaks in both the cortex and hypothalamus/preoptic area at around the third to fourth month of pregnancy (70). The particularly high enzyme activities observed in areas of the association cortex are consistent with the view that aromatase may contribute to the development of sex differences in cognitive function (53). It is striking that the highest overall aromatase activities in the cortex were observed in the prefrontal region, which is also the only cortical area assayed that appears to retain substantial aromatase activity into adulthood (65, 66). Previous studies have demonstrated sex differences in the behavioral responses of infant monkeys to lesions in the orbital prefrontal cortex, suggesting that this part of the brain develops more rapidly in males than females (32). It clearly seems possible that aromatization of circulating androgens could provide a local growth stimulus in specific areas of the developing primate cortex, resulting in regional sex differences in cortical maturation.

Similar mechanisms may also operate during the development of the hippocampus. In each of the four species that we have so far examined (rat, mouse, human, and rhesus monkey) aromatase activity has consistently been observed in hippocampal tissue samples (2, 53, 69) (figure 17.3). These findings suggest that sex differences in hippocampal morphology and function may be at least partially mediated through local estrogen biosynthesis. Previous studies have demonstrated that estrogen receptors are present in the hippocampus of rats and mice (61, 71, 72).

The role of estrogen in the differentiation of the rodent cerebral cortex remains somewhat uncertain. In organotypic cultures of newborn mouse brain, estrogen biosynthesis can be detected in the cingulate cortex using 19-OH-[<sup>3</sup>H]androstenedione as substrate. This region of the cortex contains a particularly high density of estrogen target cells at this stage of development (52, 57). In contrast, in 5-day-old rats, aromatase activity in frontal and cingulate cortex is barely detectable above the blank of the assay, well below the levels observed in either the hippocampus or hypothalamus/preoptic area at the same age. These results, which are consistent with previous studies on the distribution of aromatase in the developing rat brain (17, 18, 62), are

difficult to reconcile with the view that local estrogen biosynthesis from circulating androgens may mediate differentiation of the cerebral cortex, in all developing rodents.

It would be wrong, however, to conclude from these data that the cortex is not a target for the developmental effects of gonadal steroids, for several reasons. It remains possible that sexual differentiation of the cortex might involve aromatase-independent mechanisms—for example, effects mediated through androgen receptors (50, 65, 73). Effects of aromatizable androgens on the amygdala, hypothalamus, and hippocampus might indirectly influence the development of the cortex, through effects on afferent cortical input. The presence of estrogen receptors in the cerebral cortex of the rat well into the second week of postnatal life (48, 49) raises the possibility that circulating estrogens—perhaps derived from the ovaries, as opposed to the testes—might play a role in the differentiation of cortical function. However, such a mechanism would not explain the effects of neonatal castration on cortical asymmetry (42). It is also possible that our failure to detect aromatization in the rat cerebral cortex might reflect regional or temporal variations in expression of this enzyme, rather than a complete absence of aromatase activity. As indicated previously, aromatase is unevenly distributed in the rhesus monkey cortex and declines rapidly during perinatal development (53, 64, 65). It is conceivable that aromatase activity might change abruptly in different areas of the rodent cortex, during perinatal and early postnatal life. This could explain the apparent disparity between the results obtained in the newborn mouse cingulate cortex cultures, as compared to the postnatal day-5 rat. Detailed temporal studies on the development of aromatase activity in different regions of the rat cortex at around the time of birth will be required to resolve this issue.

Our observations on the distribution of aromatase in the developing brain raise important questions for future studies. In the hypothalamus and amygdala, aromatase activity appears to be tightly linked to the estrogen receptor system: thus, even though the brain contains relatively little aromatase in comparison to estrogen-producing organs such as the ovary and placenta, physiological levels of testosterone nevertheless result in substantial occupation of nuclear estrogen-receptor sites in the hypothalamus and amygdala (13, 60). The physiological significance of estrogen biosynthesis in the cerebral cortex and hippocampus may thus be very much dependent on whether the aromatase is localized in the receptor-containing regions of these two structures. If so, even small quantities of locally synthesized estrogen could have a major impact on the development of the estrogen target cells. A related question is whether aromatization is necessary

for the development of sex differences in cortical and hippocampal function. Although aspects of cortical maturation are clearly influenced by early estrogen exposure (35, 54–56), this does not prove that aromatization does in fact contribute to the normal androgen-induced masculinization of this part of the brain. The case for estrogen involvement would be very much strengthened if it could be demonstrated that normal sex differences in cognitive function are abolished or attenuated by blockade of aromatase activity.

In summary, studies on the distribution of aromatase activity in the brains of different mammalian species suggest that the ability to synthesize estrogen from circulating androgens is not an exclusive property of the regions of the diencephalon and amygdala in which the highest concentrations of aromatase are observed in adulthood. In the rhesus monkey, aromatase is widely distributed throughout the brain during early life, with substantial estrogen biosynthetic capacity in regions of the association cortex and somewhat lower activities in the primary motor and somatosensory regions. In mice and rats, although aromatase activity in the cortex appears to be either low (mice) or undetectable (rats) during early postnatal life, estrogen formation is clearly demonstrable in both of these species in the hippocampus, as well as in other regions of the diencephalon and limbic system. These observations are consistent with the idea that testosterone-mediated differentiation of cortical and/or hippocampal function may involve local estrogen biosynthesis.

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## Introduction

Classical steroid hormones, such as estrogen, progesterone, androgens, glucocorticoids, and mineralocorticoids, are synthesized and secreted by endocrine cells. They travel via the blood stream to their target cells, enter these cells by simple or facilitated diffusion, and then bind to specific receptors (figure 18.1). The steroid hormone receptors are intracellular transcription factors that exist in inactive apoprotein forms either in the cytoplasm or nucleus. Upon binding their respective hormonal ligands, the receptors undergo an activation or “transformation” step. The activated receptor can bind effectively to a DNA element (hormone response element, HRE) and activate transcription of a cis-linked gene (figure 18.1). In addition to regulating transcription, steroid hormones occasionally regulate gene expression by affecting mRNA stability and translational efficiency.

Although the overall pathway was proposed in the late 1960s (1–3), a detailed mechanism of action remained unclear until more recently. Since the mid-1980s, when cDNAs encoding various steroid hormone receptors were cloned, a large amount of structural information regarding steroid hormone receptors has accumulated (4). Also, the development of a cell-free system that mimicked *in vivo* receptor activity has made it possible to dissect potential molecular mechanisms of action (5–8). For discussions the identification and characterization of steroid receptors, cDNA cloning, definitions of functional domains, and identification of phosphorylation sites, readers should refer to reviews by Evans, Yamamoto, Chambon, Beato, O'Malley, Gronemeyer, and Moudgil (4, 9–15). In the present review, focus on recent data concerning receptor-DNA interactions, the role of receptors in target gene activation or repression, the role of agonists or antagonists in receptor activation, and dimeric interactions of receptors with one another or with other transcription factors that activate or silence responsive genes.

## Steroid Receptor Superfamily

The steroid hormone receptor superfamily consists of a surprisingly large number of genes (4, 16–22), and represents the largest known family of transcription factors in eukaryotes. It includes receptors for the steroids, estrogen (ER), progesterone (PR), glucocorticoid (GR), mineralocorticoid (MR), and androgen (AR). In addition, it includes receptors for thyroid hormone (TR), vitamin D (VDR), retinoic acid (RAR) and 9-cis retinoic acid (RXR), and ecdysone (EcR). Furthermore, cloning by various means has identified a large number of previously unknown genes having sequence homology to the steroid hormone receptor superfamily (22–38). Since the ligands for these genes are not known, they have been termed “orphan receptors.” Finally, an unexpected variety of isoforms of TR, RAR, RXR, ER, PR, and EcR have been identified (20, 21, 39–48). These isoforms may be expressed in distinct cell types and developmental stages, suggesting that they play a variety of physiological roles.

Amino acid sequence analysis and mutational dissection of intracellular receptors indicate that they can be subdivided into several domains as indicated in figure 18.2. The N-terminal A/B domain is highly variable in sequence and in length. Usually, this domain contains a transactivation function (AF), which activates target genes presumably by interacting with components of the core transcriptional machinery, coactivators, or other transactivators (49–53). This region may be important also for determining target gene specificity for receptor isoforms, which recognize the same response element (54, 55). The C region contains two type II zinc (Zn) fingers, which are responsible for DNA recognition and dimerization (4, 56, 57, and references therein). A more detailed description of sequence-specific recognition by the steroid receptor DNA-binding domain (DBD) and its structure is given below.

Downstream of the C region, a variable hinge region exists (D region). This region may allow the protein to bend or alter conformation, and often contains a

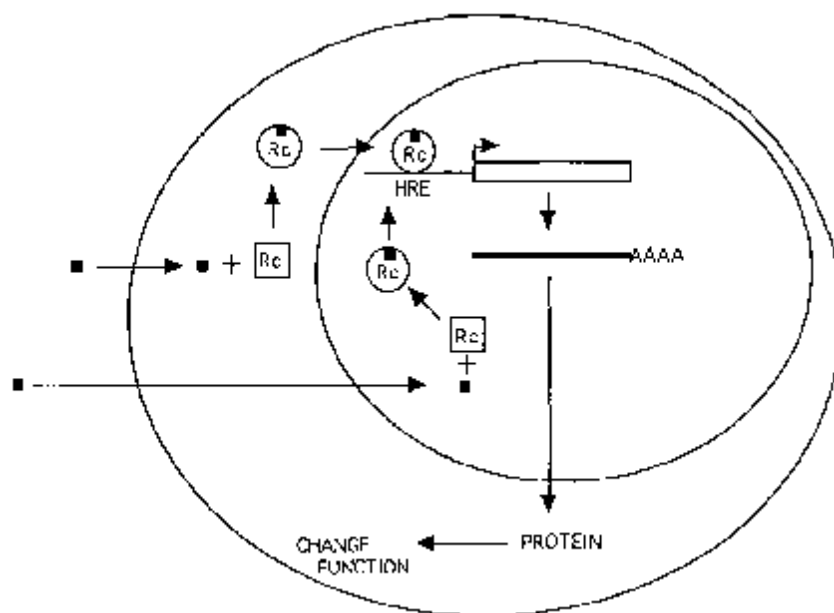


Figure 18.1

A simplified model of steroid hormone action. See text for description, *n*, steroid hormone; *Rc*, receptor; and *HRE*, hormone response element.

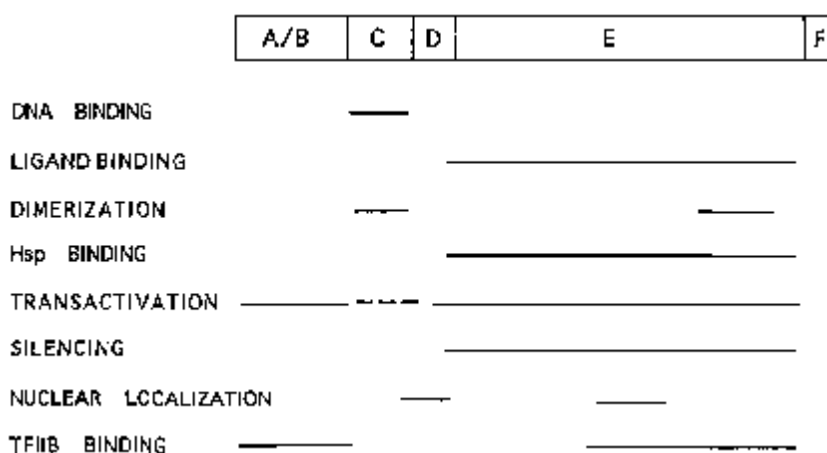


Figure 18.2

Functional domains of steroid receptors. The structure of steroid receptors can be divided into six domains: *A*, *B*, *C*, *D*, *E*, and *F*. The function of each domain is indicated by solid lines.

nuclear localization domain (GR, PR) and/or transactivation domain (TR, GR) (49, 50, 53, 58, 59). The ligand-binding domain (LBD or E region) is located carboxy-terminal to the D region. The E region is relatively large (~250 aa) and is functionally complex. It usually contains regions important for heat-shock protein association, dimerization, nuclear localization, transactivation, intermolecular silencing (TR, RAR, COUP-TF, etc), intramolecular repression (PR) and, most importantly, ligand binding (4, 51–53, 58–70 and references therein). Although most of these functions require only small stretches of amino acid sequence, ligand binding appears to involve a majority

of the E region, since most of the mutations identified in the LBD compromise the ability of the altered receptor to bind hormones. The major dimerization domain of receptors has been localized in the C-terminal half of the LBD (62, 63). This region contains leucine-rich sequences that may form coil-coil interactions as the receptor dimerizes. Finally, located at the C-terminal end of certain receptors is the variable F region, for which no specific function has been identified. For example, deletion of the F region in ER does not affect a known ER function (51).

Genomic genes for most of the steroid/thyroid hormone receptors have been cloned. For PR, GR, AR,

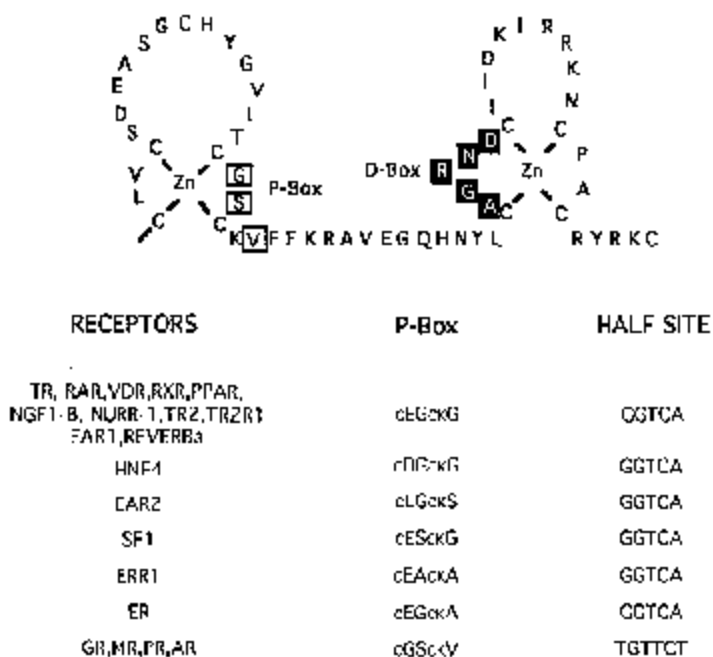


Figure 18.3

Sequence-specific recognition by steroid receptors. Amino acid sequences of glucocorticoid receptor DNA-binding domain (C) are shown. Amino acids in P-box and D-box that are important for the DNA recognition and dimerization, respectively, are highlighted.

ER, MR, TR, and RAR, the exon-intron organization is quite complex. The genes usually encompass ~60 kb and are interrupted by numerous introns. The promoter region resembles that of a housekeeping gene and is often embedded in a GC-rich island (32, 71–80). Multiple sites for initiation of transcription are the rule. In contrast, the structural genes for two orphan members of the superfamily, COUP-TF I and COUP-TF II, are more simple (81). The entire genes for these two members cover ~4–6 kb and contain only two introns. This simple organizational pattern for COUP-TF I and COUP-TF II genes may correlate with the notion that they are extreme ancestral members of the superfamily.

### Protein-DNA Interactions

DNA sequences responsive to steroid hormones have been termed hormone response elements (HREs). This concept was substantiated when an HRE for glucocorticoids (GRE) was first identified by mutational analysis of a target gene (MMTV) (9 and references therein). A deletion or mutation of that sequence eliminated hormonal responsiveness. Later it was demonstrated that a short oligonucleotide corresponding to the identified sequence conferred glucocorticoid binding and responsiveness to a heterologous promoter (82). The GRE consisted of two short, imperfect inverted repeats separated by three nucleotides. Later, response elements for progesterone, mineralocorticoid, and androgen receptors were shown to be similar to

that of GR (78, 82–87). Estrogen response elements also have been identified. Their sequence is similar to the GRE and comprises an inverted palindrome (88). Unlike GR, PR, MR, and AR, the ER does not bind to or act upon a GRE, nor does the estrogen response element (ERE) respond to GR, AR, PR, or MR. Interestingly, Klock et al (29) converted an ERE into a GRE by substituting one or two bases at the homologous position in the palindrome. Similar results were obtained by Martinez et al (89).

By the same token, conservation among HREs suggests that the receptor amino acids responsible for DNA binding must be conserved, so that only minor change(s) in the amino acid sequence of the DBD might change the specificity of DNA binding. Indeed, Mader et al (97) have identified three amino acids in the so-called P-box (EGckA) that are essential for ERE recognition (figure 18.3). Mutation of these amino acids to GSckV changes ERE recognition to GRE recognition. Similar observations were made by Danielsen et al (91) and Umesono and Evans (92), who generated a version of GR that bound functionally to an ERE/TRE by changing amino acids in the P-box. Using the P-box sequence as an indicator of DNA recognition specificity, Forman and Samuels (63) classified receptors into several groups (figure 18.3). It is clear that the P-box sequence cGSckV recognizes the TGTTCT half site, while cEGckG, cEGckS, cEAcckA, cESckG, cDGckG, or cEGckA P-boxes recognize the AGGTCA half site. Since receptors prefer to bind to

DNA as dimers, it may be expected that the recognition sequence is a repeat, either direct or inverted, depending on receptor origin and on whether the receptor binds as a homo- or heterodimer (see below).

The structure of the receptor DBD was first solved by NMR and later refined and confirmed by X-ray crystallographic analysis (56, 93–96). The DBD of the GR is a globular structure that can be subdivided into two modules. Each module consists of a Zn coordination center and an amphipathic  $\alpha$  helix. The first module contains the first Zn finger. It starts with a short segment of antiparallel  $\beta$  sheet and ends with an  $\alpha$ -helical structure between the second pair of Zn coordinating cysteines. The  $\beta$  sheet helps to orient the residues that contact the phosphate backbone of DNA. The helical structure (P-box and downstream amino acids, figure 18.3) provides important deoxynucleotide contacts and fits into the major groove of the DNA helix. The second module is more important for phosphate contacts and for dimerization (D-box between A476 and D481) of the two DBD molecules. The two modules form a globular structure through the interaction of aromatic side chains of conserved amino acids in the amphipathic helices (F463, F464, Y497, Y452, and Y474, figure 18.3).

A similar structure was obtained for the DBD of ER (94, 95) except that the ER structure is at somewhat higher resolution and allows one to address the question of how ER and GR discriminate between their binding sites. While the GR-specific amino acid Val29 contacts the GRE-specific thymidine base of TG(T)TCT, the ER-specific amino acid Glu25 interacts with the cytidine of TGA(C)C. Surprisingly, the conserved amino acid Lys32, which plays no role in GR interaction with its GRE, interacts with central G-C and T-A base pairs that are specific for an ERE. To some degree, these results confirmed earlier results obtained by biochemical and mutational analyses (91, 92, 97), which suggested that amino acids in the P-box were responsible for base recognition and discrimination. Also, prior studies had indicated that the DNA-binding domain of GR binds to a GRE preferentially as a dimer (98, 99), suggesting the existence of a dimerization function the DNA-binding domain.

While GR, PR, ER, AR, and MR bind to DNA as homodimers and recognize a palindromic response element, other receptors such as TR, RAR, VDR, RXR, PRAR, ultraspiracle, and COUP-TF can recognize direct repeat response elements (100–102). This latter group of receptors can form heterodimers with each other. More importantly, TR, RAR, VDR, and PPAR bind to their cognate DNA responsive elements with higher affinity as heterodimers with RXR than as homodimers (69, 103–111). Consequently, it has been predicted that the heterodimer is the major functional

complex for this group of receptors. Similarly, the ecdysone receptor functions by heterodimerizing with ultraspiracle, a functional homolog of RXR from *Drosophila* cells (112).

Since TR, RAR, VDR, COUP-TF, PPAR, and RXR all bind to direct repeats of AGGTCA, a question could be raised as to whether any binding-site discrimination occurs among these receptors. Using AGGTCA direct repeats with various spacers, Umesono et al (100) and Naar et al (101) have derived the so-called “3,4,5 rule.” DR-1 (AGGTCA direct repeat with one nucleotide spacer) can bind and act as a response element for the RXR homodimer, COUP-TF homodimer, RXR-COUP-TF heterodimer, and PPAR homodimer or heterodimer with RXR. DR-3, DR-4, and DR-5 serve as response elements for VDR, TR, and RAR, respectively. Also, DR-2 has been shown to bind to TR. Although the “3,4,5 rule” appears generally correct, receptors in this category may bind to and activate each other’s response elements, albeit at lower efficiency.

Promiscuous binding and gene regulation by receptor family members may be more frequent than expected. For example, COUP-TF can be considered the “plastic man” of this superfamily. It binds as a homodimer with reasonable affinity to the GGTC A direct repeat with a spacer of anywhere from 1 to 10 nucleotides (102); it can bind also to inverted repeats with different spacers. This promiscuous binding indicates that the two DBDs of the COUP-TF dimer are not fixed in the dimeric structure. Instead, they are flexible and can be induced by the DNA to swivel and accommodate different spacers and orientations between the two half sites on the DNA target element. This conclusion is supported by studies showing that COUP-TF binds across various response elements differently (113). It wraps around the COUP sequence of the ovalbumin gene, but only contacts one face of the DNA response element (RIPE-1) of the insulin gene (113). The functional consequence of the promiscuous nature of COUP-TF binding to GGTC A repeat sequences is reflected in its ability to bind a variety of response elements and negatively affect the activity of many other receptors (69, 102, 111, 114).

A three-dimensional structure for this subgroup of receptors has been determined only for the RXR DBD. In addition to the two amphipathic  $\alpha$  helices of the GR and ER DBDs mentioned above, RXR contains another  $\alpha$ -helix immediately after the second Zn finger (96). Mutational analysis indicates that this helix is necessary for both DNA recognition and homodimerization. The requirement for an additional helix for dimerization is not fully understood. However, since RXR binds to direct repeat elements, this additional helix may be required to hold two RXR DBDs in a

head-to-tail configuration. If that is the case, COUP-TF and TR, which can bind to both direct and inverted repeat response elements (102), also may have differential requirements for a third helix. Additional questions regarding the mechanism by which RXR forms heterodimers with TR, RAR, PPAR, and VDR remain to be resolved. Considering the rapid progress in X-ray crystallographic studies of the receptor DBDs, the structure of a heterodimer should be forthcoming.

In addition to receptor dimer binding to DNA repeat elements, several orphan receptors appear to recognize their response elements as monomers. RevTR $\alpha$  (EAR1) and NGFI- $\beta$  recognize the AGGTCA sequence without repetition (115, 116). However, additional AT-rich sequences upstream of the AGGTCA sequence are also important for this recognition (116).

While most receptors can bind *in vitro* to their cognate DNA response elements in the apparent absence of a ligand, steroid hormone receptors usually do not bind to DNA in the absence of ligand *in vivo*. Furthermore, PR and ER can be demonstrated to bind to DNA in a hormone-dependent manner *in vitro* (117–120). Similarly, induction of homodimer binding to DNA by the RXR ligand (9-*cis*-retinoic acid) has been reported (121).

The mechanism by which ligands induce steroid receptors to bind DNA is of interest. It has been proposed that the ligand-binding domain blocks the DBD in the absence of a ligand; subsequently, ligand binding changes the structure and exposes the DBD. Also, since this group of aporeceptors is thought to exist in a complex with heat-shock proteins (hsp90, hsp70, and hsp56) prior to exposure to ligand, it has been proposed that these heat-shock proteins interfere with DNA binding (60, 122). Binding of ligand is thought to disperse heat-shock proteins, relieving the inhibition. Nevertheless, these two hypotheses are not consistent with the observation that the ligand-binding domain of ER or GR can be fused to heterologous transcription factors in different positions and can render these transcription factors ligand-dependent (122–125). One would predict that if the ligand-binding domain or heat-shock proteins simply block DNA binding, the ligand-binding domain located at different distances or positions from the DBD should have quantitatively different effects on receptor-DNA interaction. Furthermore, removal of heat-shock proteins by biochemical means does not make human PR bind DNA constitutively (126); ligand is still required for inducing DNA binding by the progesterone receptor free of heat-shock protein. These results suggest that ligands must play an additional role to induce steroid receptors to bind to DNA.

Recently, Allan et al took another approach to answer this question. This approach took advantage of

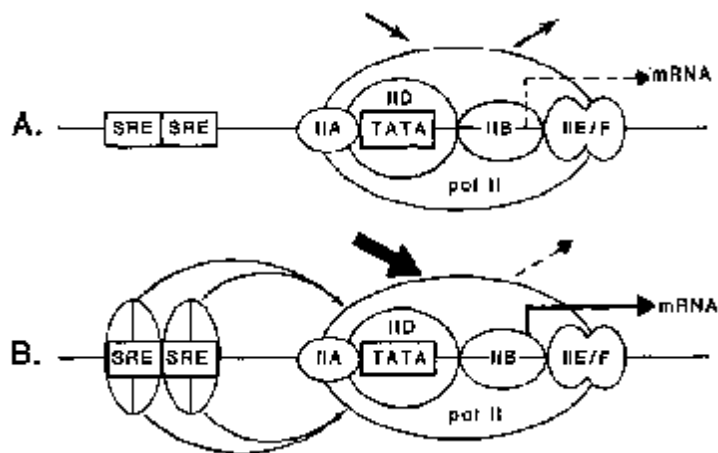
the observation that GR (also PR and ER) binds to DNA as a dimer (98, 127, 128). The monomeric form of the receptor (DBD only) binds to DNA with sufficiently lower affinity that one must use purified receptor and low levels of nonspecific competitor DNA in binding assays in order to detect binding by gel mobility shift assays. Since it is possible that the lack of detection of DNA binding in the absence of ligand is due to an inability of receptor to form a dimer, Allan et al used an antibody as the dimerization agent (one divalent antibody forces two receptor molecules to bind to DNA at adjacent sites) in the absence of ligand (129). Indeed, divalent monoclonal antibodies that recognize human PR induced the receptor to bind to its response elements, while control antibody did not. Thus, the role of ligand in DNA binding is likely to be induction of a conformational change in the ligand-binding domain (discussed below), which exposes the major dimerization function present in this region. The dimeric receptor can then bind to its HRE with high affinity. This conclusion is consistent with the ability of other nuclear receptors such as TR, RAR, COUP-TF, and VDR to dimerize in the absence of ligand and thereby bind to DNA constitutively (100, 101).

## Role of Receptor in Gene Activation and Silencing

### Gene Activation

Upon binding hormone, receptors bind to DNA and activate target gene expression. Progress in understanding the mechanism of transactivation was hampered by the lack of appropriate *in vitro* systems. Several years ago, a cell-free transcription system that allowed one to demonstrate activation of target genes by exogenous purified steroid receptor was developed (5–8). Similarly, baculovirus-expressed GR, PR, and ER were shown to activate target genes (118, 130–132). Activation was observed with minimal as well as naturally occurring promoter constructs (133). The ability of steroid receptors to activate minimal promoter constructs suggested that activation might occur via direct interaction of the receptor with the core (TATA) transcriptional machinery. Indeed, using template commitment assays *in vitro*, we demonstrated that cPR, mER, and hGR activate transcription by enhancing the formation of stable preinitiation complexes at their target promoters (6, 130, 132).

A simplified illustration of receptor induction of transcription at a typical target gene is summarized in figure 18.4. This scheme proposes that the receptor stimulates the formation of a preinitiation complex by increasing its rate of formation and/or by stabilizing a preformed preinitiation complex. The action of receptors to stimulate preinitiation complex formation could be either by direct interaction with components of the

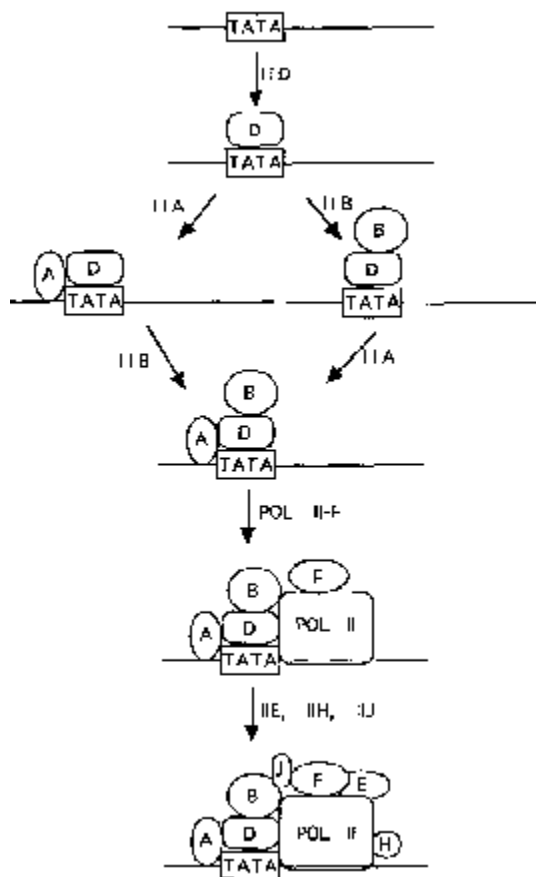
**Figure 18.4**

Steroid receptor stabilization of the preinitiation complex for transcription. See text for description.

transcriptional machinery or through an intermediate factor such as a coactivator. Formation of the preinitiation complex at a core promoter is a sequential process (figure 18.5). First, TFIID recognizes the TATA box; for a TATA-less promoter, binding of an initiator protein may be required. Next, TFIIA and TFIIB bind independently to the TFIID-DNA complex. Importantly, when TFIIB enters the complex, the TFIIF-RNA polymerase complex can then enter to form the ABDF-polymerase-DNA complex. Lastly, the remaining general transcription factors assemble to form the complete preinitiation complex (for review see ref. 134). It is possible that a receptor could act on any of these steps to enhance formation of the stable preinitiation complex.

A hint at which step the receptor enhances gene transcription came from earlier studies that indicated that COUP-TF, a member of the steroid/thyroid hormone receptor superfamily, interacted specifically with a transcription factor that we called S300-II (135). After cloning of the S300-II factor, it was found to be TFIIB (136). This interaction probably does not require a helper protein, since renatured COUP-TF from a single gel band was able to bind specifically to TFIIB. Similar observations were made for PR, ER, TR, and RAR (136, 137; XH Leng, SY Tsai, M-J Tsai, BW O'Malley, unpublished results). These results are consistent with results obtained using other activators, such as VP16 (138) and Ftz protein (139). VP16, a viral transactivator, has been shown most clearly to activate target genes by interacting with TFIIB. Mutation of amino acids necessary for the VP16-dependent activity of TFIIB but not for its basal activity destroys the specific interaction between TFIIB and VP16 (140).

Using TR as a model, Baniahmad et al (137) have found that the N-terminal end of the receptor interacts specifically with TFIIB. Like VP16, this interaction

**Figure 18.5**

Steps in assembly of the preinitiation complex for transcription. See text for description.

requires the second half of TFIIB, which contains a direct repeat. Since binding of TFIIB to the TFIID-DNA complex is one of the rate-limiting steps in preinitiation complex formation, it is plausible that through this interaction TR enhances its formation. It should be emphasized, however, that the receptor may also interact with other components of the transcriptional machinery. TBP (a subunit of TFIID) has been shown to interact with TR and other transactivators (141, 142; A Baniahmad, SY Tsai, M-J Tsai, BW O'Malley, unpublished results). However, since TBP is a relatively sticky protein, the significance of this interaction remains to be determined.

### Gene Silencing

Classical steroid receptors have been considered to function *in vivo* only in the presence of their hormonal ligands. In the absence of ligands these receptors cannot bind to DNA, and thus remain functionally silent. In contrast, the subgroup of smaller nuclear receptors including TR can bind to DNA in the absence of ligand (see below). Although in most cases they cannot activate target genes, they are not neutral in regulating target gene expression. In the absence of hormones, they frequently silence basal promoter activity (64, 66–69, 143). The region important for the silencing activity is localized within the C-terminal hormone-binding domain. Within this domain, both the proximal and distal C-terminal regions are required (67). Consequently, the LBD can be subdivided into two halves, each of which has no silencing activity by itself. In contrast, cotransfection of both halves into cells restores silencing activity (A Baniahmad, SY Tsai, M-J Tsai, BW O'Malley, unpublished data). Since silencing activity requires a DNA-binding site, inhibition of basal promoter activity is unlikely to be due to cellular squelching of a coactivator. Furthermore, silencing activity can be detected with a simple promoter construct containing only a TATA box and an HRE (137), suggesting that silencing is a result of direct interaction of the receptor with the core transcriptional machinery.

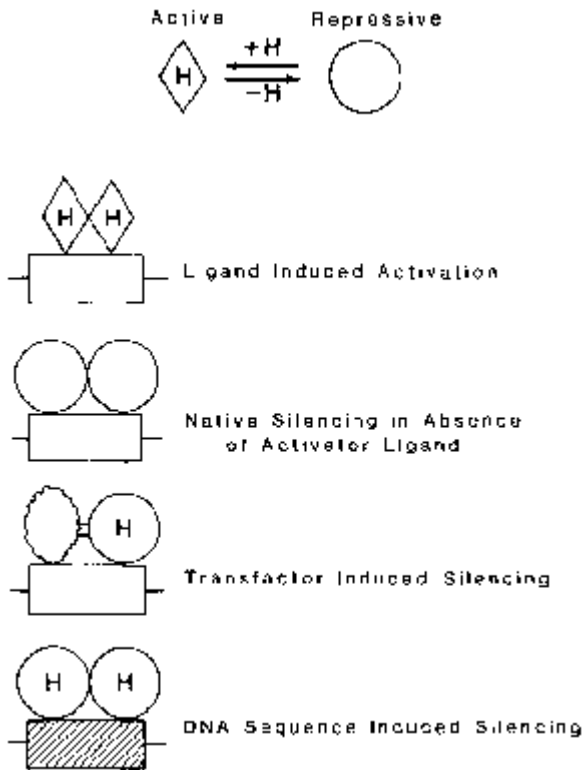
Recently, Fondell et al (144) have duplicated silencing activity *in vitro* using TR expressed in *Escherichia coli*. They have demonstrated that the silencing activity of TR is due to its ability to inhibit the formation of a preinitiation complex. They demonstrated that the receptor can interact with TFIIB, but in their *in vitro* system the ligand (T3) was unable to convert the receptor from a silencer to an activator as happens *in vivo*. Recent evidence to support the notion that this interaction with TFIIB is important for silencing activity came from work carried out by Baniahmad et al (137). It was demonstrated that TR interacts with TFIIB specifically in two distinct regions. The N-terminal do-

main of the receptor and the C-terminal half of the ligand-binding domain can interact separately with TFIIB. The N-terminal region of TR interacts with the C-terminal half of TFIIB and, as discussed earlier, this interaction may be important for transactivation activity. In contrast, the C-terminal domain of TR interacts with the N-terminal Zn finger of TFIIB, which is important for basal activity. Importantly, this interaction is sensitive to hormone. In the presence of physiological concentrations of T3, binding is decreased significantly. Since this region of TR is important for silencing and since the interaction with TFIIB is sensitive to ligand, it was proposed that the interaction is functional for silencing in the absence of ligand. Since the Zn finger region of TFIIB is important for basal activity due to its interaction with the RNA polymerase–TFIIF complex (via RAP30 subunit of TFIIF) (145), it is tempting to speculate that competition for binding to the putative Zn finger of TFIIB between the TR ligand-binding domain and the TFIIF-RNA polymerase complex is the underlying mechanism for silencing by TR. Finally, similar results have been obtained with RAR; addition of retinoic acid decreases the strength of specific binding between RAR and TFIIB (XH Leng, SY Tsai, BW O'Malley, M-J Tsai, unpublished observation).

Recent evidence has provided a strong argument for the existence of negative HREs, at least in the case of the glucocorticoid receptor (146, 147). These are specific DNA sequences that allow high-affinity binding of receptor but that appear to force it into a conformation that exposes peptide domains that silence transcription. It had been thought previously that silencing activity was likely to be induced solely by adjacent *cis* elements occupied by *trans* factors, which exert their negative combinatorial effects via protein-protein interactions with receptors (figure 18.6). This appears not to be the case, since glucocorticoid receptor silencing activity is seen with unrelated combinations of positive activators and appears to require only the core transcriptional machinery located at the TATA promoter for its negative impact. A summary of some of the known mechanisms by which receptors may silence or repress target genes is shown in figure 18.6.

### Synergism between Different *cis*-Acting Elements

Many eukaryotic genes are under the control of multiple hormones and environmental cues. Consequently, steroid hormone response elements are usually found in multiple copies or clustered with other *cis*-acting elements. The tryptophan oxygenase gene, the MMTV LTR, and the vitellogenin genes contain multiple HREs (148–150). In addition, the MMTV LTR, rat tryptophan oxygenase, and phosphoenolpyruvate

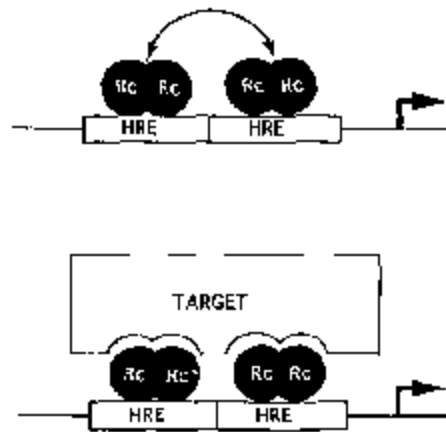


**Figure 18.6**

Conformational states of steroid/thyroid receptors. Models depict different modes of action by steroid hormone receptors to activate or repress target gene expression. See text for description.

carboxykinase gene contain other *cis*-acting elements in close conjunction with the HREs (149, 151, 152). In these cases, when either one of the HREs or the adjacent *cis*-acting elements is mutated, promoter activity is drastically decreased. Thus, HREs often interact synergistically with other HREs or with unrelated *cis*-acting elements. Using artificial reporter genes in transfected cells, one can demonstrate synergistic activation when multiple HREs or an HRE together with other *cis*-acting elements are inserted in front of a target gene (150, 153–157).

As depicted in figure 18.7, Ptashne has proposed two models to explain synergism that are relevant to receptor superfamily members (158). First, binding of transcription factor dimers to two or more DNA response elements can be cooperative. Binding of one receptor complex facilitates the binding of a second (figure 18.7, *top*). This synergistic interaction allows both complexes to bind with greater affinity and consequently in greater occupancy of the *cis*-acting elements, thus promoting more transcription. This type of synergism would be observed only if the transcription factors are in limited concentration, and indeed, cooperative binding of receptors to multiple response elements has been observed with PR, GR, and ER (154, 159, 160). It is



**Figure 18.7**

Synergism between two HREs. Model depicts how two HREs could synergize in a target gene activation. Models that depend upon cooperative binding of receptor dimers to DNA (*top*) and synergistic interaction of receptor dimers with a target on the transcription machinery (*bottom*) are shown.

likely that protein-protein interactions between the two dimers facilitate cooperativity. Tsai et al found that two molecules of the *E. coli*-expressed DBD of GR were able to bind to a single response element in a cooperative manner (98). However, two dimers of GR DBDs were not able to bind cooperatively to two adjacent GRE/PREs, but the full-length receptor could do so (154; SY Tsai, M-J Tsai, BW O'Malley, unpublished observation). Since both the DBD and the full-length receptor can bind DNA, cooperative binding between two receptors appears to require protein-protein interactions outside the DBD domains. By deletion analysis (155), the region required for cooperative binding was localized to position 250–417 of cPR, which includes the DNA-binding domain (amino acids 280–369).

Although cooperative binding is an attractive model, it is unlikely to account for the total level of transcriptional synergism observed at target genes, especially when different HREs are present. For example, a PRE and an ERE can activate transcription of a linked target gene synergistically, but very little cooperative binding between PR and ER can be observed (155). The second mechanism proposed in figure 18.7 (*lower model*) suggests that cooperative interactions of receptors or transcription factors with multiple target sites (presumably components of the aggregate transcriptional machinery) may play a role in synergism. Attempts to define the regions important for this second type of cooperativity have proven to be unsuccessful. Mutation of a variety of regions in PR decreases its ability to synergize with ER (155). Similarly, synergistic interaction has been observed between GR and other transcription factors, and the structure important

for this interaction has been shown to be complex (156).

### Role of Ligand in Receptor Transformation and Activation

#### Role of Ligand

Intracellular hormone receptors have either neutral or silencing activity in the absence of ligand. Upon binding their cognate ligand, they usually become positive regulators, although certain genes can be repressed if the cis-elements are arranged appropriately. The receptor activation process is called transformation. Members of the receptor superfamily can be roughly divided into two subgroups according to their functional properties. Group A consists of the larger steroid receptors GR, AR, PR, MR, and ER, which have longer A/B domains. In the absence of hormone they have been shown to exist as 8–10S complexes associated with heat-shock proteins (hsp90, hsp70, and hsp56) (161–169). For the most part, unliganded group A receptors do not bind to DNA and thus have neither transcriptional nor silencing activity. Upon binding hormone, heat-shock proteins dissociate, the receptors sediment as a 4S complex, and they are able to dimerize, bind to DNA, and transactivate a target gene. In contrast, Group B receptors such as TR, RAR, VDR, RXR, PPAR, and most (if not all) of the orphan receptors have short A/B domains. They are nuclear receptors and have not been shown to be associated with heat-shock proteins. They appear to be able to bind to DNA in the absence of ligand. Upon binding hormone, all receptors appear to undergo transformation to activators. Although there are distinguishing features between the two groups of intracellular receptors, the high degree of conservation in both the DNA- and ligand-binding domains suggests that the underlying mechanism of hormonal regulation is similar. In this section we discuss hormonal control of these groups of receptors separately, then we offer a unified model to resolve their distinguishing features.

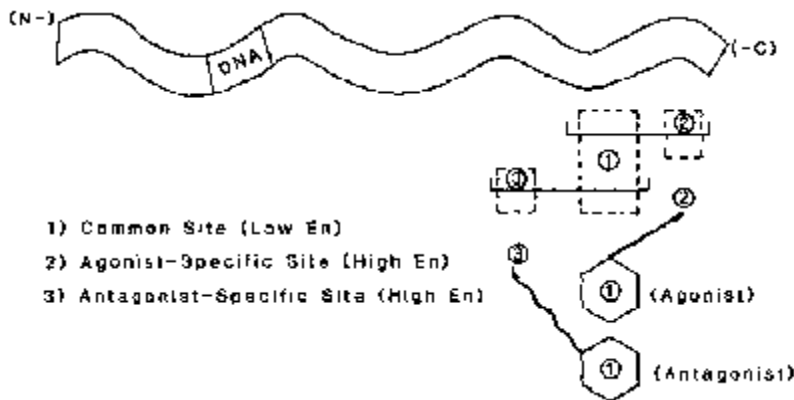
Due to the observation that ligand dissociates heat-shock proteins from a Group A receptor coincident with activation, it was considered that the role of the ligand is simply to remove the inhibitory heat-shock proteins, thus allowing DNA binding and transcription (60, 122). In this scenario, the receptor should be constitutively active after heat-shock protein removal. Picard et al, using a yeast genetic system to reduce the cellular level of hsp90, found that the response of GR to its ligand had been altered (170). However, receptor activity remained dependent on exogenous ligand. These results argue that removal of hsp90 itself is not sufficient for receptor transformation. It is not clear from these experiments, however, whether other receptor-associated heat-shock proteins, including

hsp70 and hsp56 or a residual amount of hsp90, had any effect on the hormone dependency.

Direct evidence that removing heat-shock proteins is not sufficient to convert a receptor from a hormone-dependent to a constitutively active molecule came from *in vitro* studies (126). Human PR from T47D cells was used to develop a hormone-dependent DNA-binding and transcription system (5, 117). Using this hormone-dependent, cell-free system and purified receptor free of hsp90, hsp70, and hsp56, it was demonstrated that the heat-shock protein-free receptor still binds to DNA and transactivates a target gene in a hormone-dependent manner. These results argue strongly that the steroid hormone plays an additional role(s) beyond removal of heat-shock proteins.

One can envision two possible roles that hormones may have in receptor activation. First, the receptor may require covalent modification (e.g. phosphorylation) for DNA binding and transactivation. Indeed, ligand-dependent phosphorylation has been observed with PR (171–173). However, as is discussed below, a great deal of phosphorylation occurs after the receptor binds to DNA (174–176). Furthermore, removal of receptor phosphates by alkaline phosphatase treatment did not prevent hormone-dependent binding to an HRE (G Allan, SY Tsai, M-J Tsai, BW O'Malley, unpublished observation). Thus, while it may be important for maximal gene transactivation activity, phosphorylation of receptor is not absolutely required for ligand-dependent activation of receptor.

The second possibility is that a conformational change is induced by hormones. It has been observed in a number of laboratories that upon binding hormone, certain steroid receptor-DNA complexes migrate to a different position from the ligand-free receptor in a gel mobility shift electrophoretic assay (118, 177–179). In addition, Fritsch et al showed that hormone-bound estrogen receptor partitioned in PEG-palmitate differently from the unliganded receptor (180). These results suggested that the receptor may have a different conformation after binding ligand. In order to show more directly that this is the case, Allan et al used protease digestion and monoclonal antibody mapping as probes to detect ligand-dependent conformational changes (181). It was reasoned that if ligand induced a different conformation in the receptor, protease digestion sites may become more or less sensitive to digestion, depending on their location. Indeed, it was found that PR is generally quite sensitive to protease digestion in the absence of ligands. Upon binding progesterone or other agonists, a portion of the receptor becomes very resistant to protease digestion and a ~30-kDa protease-resistant fragment appears. Resistance to protease digestion appears due to a major structural change, since resistant bands of about the



**Figure 18.8**

Two-step binding of ligand to progesterone receptor. This model argues that agonists and antagonists of the progesterone receptor recognize distinct regions of the ligand-binding domain. It is suggested that each may interact initially with a low-affinity site and then make high-energy (high-En) contacts with different specific binding sites, consequently inducing different C-terminal structures.

same size were observed regardless of what proteases were used. Thus, progesterone induces a conformational change converting virtually the entire ligand-binding domain (E region, figure 18.2) to a very compact structure. The conformational change was confirmed also by monoclonal epitope mapping in this region (70, 182).

The protease-resistant conformational change occurs before heat-shock proteins are released from the PR (181). In fact, it is likely that this conformational change induces their dissociation from the 8–10S receptor complex. In the absence of heat-shock proteins and with the new conformation in the ligand-binding domain, the receptor is able to dimerize and bind to target DNA response elements.

The observation that protease-resistant conformations are induced in receptors by hormones has been extended to estrogen, glucocorticoid, and androgen receptors (118). Importantly, experimental conditions that induce the protease-resistant conformation in the absence of ligand also create a receptor that is active in transcription. Thus, it is speculated that this conformation is essential for a receptor to carry out transactivational regulation.

Substantial new evidence on the mechanism of action of hormone antagonists comes from recent studies (181). Previously, it was believed that hormone antagonists are compounds that can bind competitively with authentic agonists at the identical site on their cognate receptors. Recent evidence has changed our appreciation of the sites at which agonistic and antagonistic ligands bind to the LBD of intracellular receptors. Mutational analyses have clearly established that the agonist- and antagonist-binding sites on the human PR are distinct (70, 183). Rather than agonists and antagonists competing for the identical sites, they appear to compete for agonist- or antagonist-induced structures.

For example, agonists bind most tightly to the extreme C-terminal tail of the LBD, while antagonists make their high-affinity contacts at a more proximal region, as indicated in figure 18.8. Variations on this theme may apply also to the estrogen receptor (184) and glucocorticoid receptor (183).

Most antagonists are able to stimulate receptors to bind to DNA and to undergo phosphorylation (117, 175, 179). Nevertheless, the antagonist-bound receptor is not active in turning on target genes, although some antagonists have partial agonist activity. A question can be raised as to why an antagonist is able to promote most of the events observed with an agonist, yet the DNA-bound receptor is transcriptionally inactive. From the above discussion, we would predict that an antagonist must also cause conformational change to expose the dimerization domain, allowing the receptor to bind to DNA with high affinity. Nevertheless, this conformational change must differ functionally from that induced by the agonist. This hypothesis is consistent with the observation that an antagonist-bound receptor-DNA complex migrates differently electrophoretically from that of an agonist-receptor-DNA complex (118, 177–179). More direct evidence came from Allan et al (181). It was observed that like the agonist-bound receptor, the antagonist-bound receptor is resistant to protease digestion, but the resistant fragment is slightly shorter than that of the agonist-bound receptor (27 vs 30 kDa). The results suggest that antagonist binding induces a conformational change in a receptor that is different from that induced by an agonist. A similar conclusion has been drawn from antibody studies (70, 182). It was found that an antibody that recognizes the C-terminal tail of PR recognizes the receptor when it is ligand-free or antagonist-bound. However, it cannot recognize the epitope when the receptor is bound to an agonist.

The difference between agonist- and antagonist-induced conformations has been localized to the extreme C terminus of the LBD. The protease digestion site 3 kDa upstream of the C-terminal end is available to the protease when the receptor is complexed with an antagonist, but the same site in the agonist-bound receptor is hidden from the protease. Therefore, we reasoned that the sequences within the final 3 kDa of the PR must play a role in distinguishing between agonist and antagonist activity. Functional evidence for the role of the C-terminal end comes from studies by Vegeto et al, who used yeast as a system to generate mutants that have altered ligand specificity (70). Using this approach, a PR mutant termed UP-1 was generated. This mutant was no longer responsive to progesterone agonists but was activated by progesterone antagonists such as RU486. Sequence analysis indicated that the UP-1 mutation results from a frameshift replacing the last 54 amino acids of the receptor. More detailed studies showed that the last 23 amino acids are required for progesterone binding but that the last 54 amino acids are not needed for RU486 binding (G Allan et al, unpublished observation). The results indicate that the C-terminal end of PR contains a repressor function that downregulates the activity of aporeceptor or receptor bound to an antagonist. Inactivation of this repressor function by deletion creates a receptor that is now able to activate a target gene in the presence of antagonists.

These results are consistent with the conclusion drawn from protease digestion studies, which indicated that the C-terminal end of PR plays an important role in determining antagonist activity. Similar differential protease digestion patterns were observed with ER and GR, suggesting that a related repressor function might also exist in these receptors. However, with these receptors the story may be somewhat more complicated, since a simple deletion of C-terminal amino acids results in similar decreases in receptor activity for both agonist and antagonist in each case (185; G Allan et al, unpublished observation). Nevertheless, the hypothesis is consistent with work described by Danielian et al (186), who also demonstrated the existence of a transactivation domain in the C-terminal region of ER. Strong evidence for a repressor function in GR comes from a report by Lanz et al (185), who showed that point mutations of two hydrophobic amino acids (770 and 771) and deletion of another pair of conserved amino acids (780 and 781) present in the C terminus resulted in a receptor responsive to the antagonist, RU486, but not to the agonist, dexamethasone. Similarly, an androgen receptor isolated from LNCap cells, which has a mutation at amino acids 868 (Thr to Ala) is activated now by anti-androgens (187). Taken together, these results support the general

existence of repressor functions within the C terminus of the steroid receptor superfamily members.

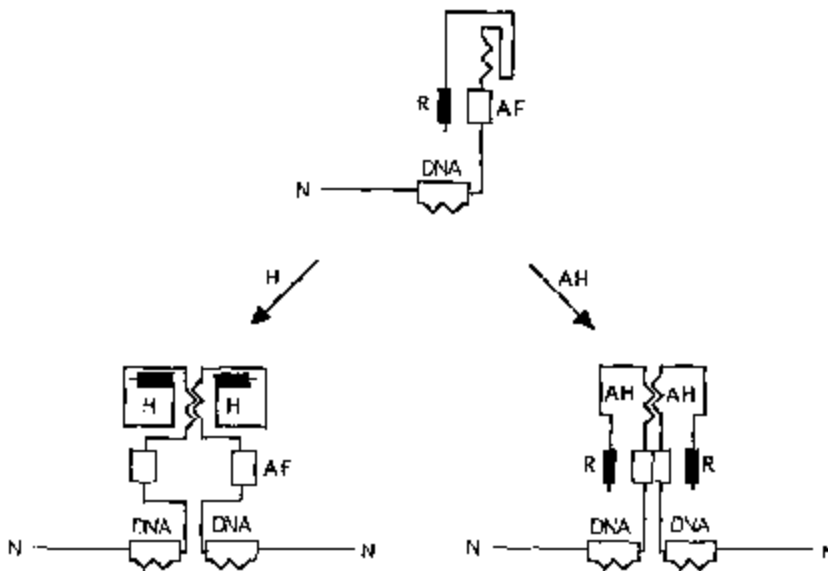
The identification of an intramolecular repressor function in Group A receptors raises the question of whether the repressor function of the Group A receptor is related to the silencer function of Group B receptors. It is interesting to note that both of these two functions are localized within the C-terminal end of the molecule. Exchange of the C-terminal sequence between these two groups of receptors could answer this question.

### Model

The hypothetical model presented in figure 18.9 illustrates our current hypothesis for activation of a Group A steroid receptor by a hormonal agonist (H) or antagonist (AH). In the absence of ligands, the receptor exists in a conformation such that the DBD could be available for DNA binding. However, the dimerization domain is not available, so the receptor cannot bind to DNA with high affinity. Furthermore, the activation domain(s) (AF) is under the control of the intramolecular repressor (R), and therefore, is unable to activate transcription. Upon binding agonist (H), the receptor alters conformation, resulting in the exposure of the dimerization domain; the receptor dimerizes then and binds to DNA. In parallel, the R domain is sequestered from the surface of the receptor by the hormone (since the R region is required for agonist binding) into a compacted structure that is not available to protease or antibody and thus is not able to continue repressing the AF(s). Without inhibition by the R region, the AF can interact with its target, presumably components of the transcriptional machinery, to activate gene transcription. Since there are multiple AFs in a given receptor (49–53, 64–66), it is possible that only the C-terminal AF in the hormone-binding domain is under the R regulation. This possibility may explain why some antagonists have partial agonist activity, presumably by utilizing other AFs in the N terminus. It should be noted also that inhibition of AF by the R region is not necessarily as depicted in figure 18.9. It could either block the AF by physical means, compete for the target component, or serve as a binding site for a distinct repressor protein.

In contrast, when the receptor binds to the antagonist (AH), there is an incomplete conformational change, also resulting in exposure of the dimerization domain. Thus, the receptor can dimerize and bind to DNA. In contrast, the C-terminal R region is still available for protease and antibody recognition and to interfere with the AF. Thus, while the antagonist-occupied receptor can bind to DNA, it is not able to activate gene transcription.

The Group B receptors have certain distinguishing features. They are able to bind to DNA in the absence



**Figure 18.9**

Mechanism of action of agonist and antagonist ligands. A key point is that an antihormone (AH) does not induce a structure that removes a surface repressor function. See text for description. H, hormone; AH, antihormone; AF, activation function; R, repressor function; DNA, DNA-binding domain; ~, dimerization domain; N, N terminus.

of ligand. The lack of heat-shock protein association with this group of receptors may contribute to their ability to bind DNA without a ligand. Also, certain receptors in this group (e.g. TR) may bind to DNA as monomers. However, it is more likely that their ability to form a homodimer or heterodimer (with RXR) in the absence of hormone is the key feature that allows them to bind to DNA with high affinity (69, 100, 101, 103–111). The fact that they cannot activate target genes even though they are bound to DNA may again relate to conformational studies obtained by protease digestion. Leng et al (188) found that in the absence of ligand, TR and RAR are sensitive to protease digestion. Ligands also make the ligand-binding domains of these receptors protease resistant. Thus, the story with this group of receptors is similar to that of Group A: The protease-resistant conformation is required for receptor to be able to activate transcription. Recently, Toney et al (189) demonstrated a ligand-dependent conformation change in the TR ligand-binding domain using circular dichroism.

Figure 18.10 summarizes the overall pathway for steroid/thyroid hormone receptor activation of target genes. For Group A receptors, the conformation changes upon hormone binding, and results in the dissociation of hsp, dimerization, and binding to target HREs. After phosphorylation by a DNA-dependent protein kinase (discussed below), the receptor is able to facilitate the formation of the preinitiation complex and induce gene transcription. Group B receptors, which are bound to DNA as dimers, await conformational activation by ligand in situ. Transcriptional

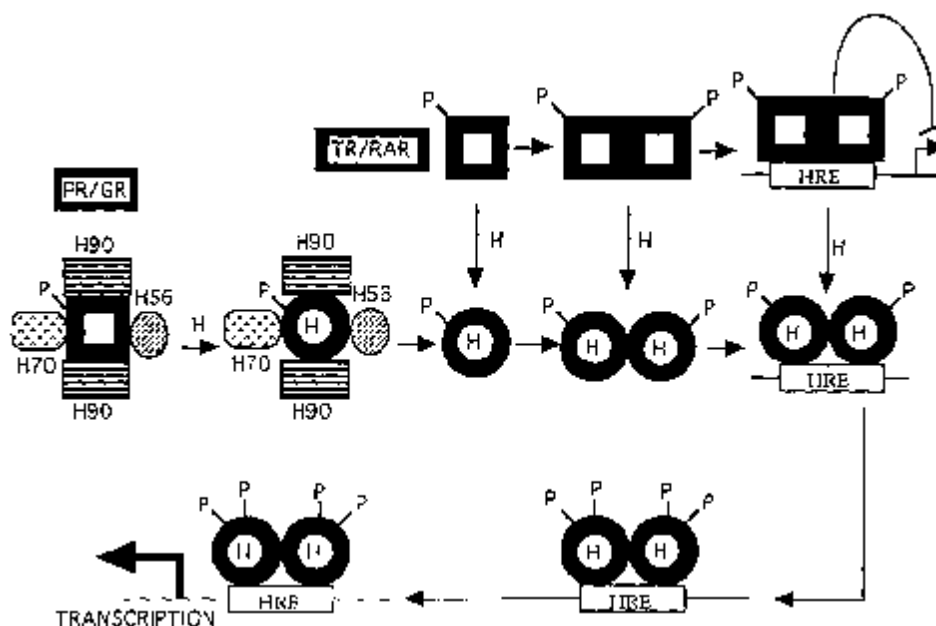
effects follow. Further studies are needed to determine if the silencing function of Group B receptors is identical to the repressor function of Group A receptors and to substantiate the apparent ligand-induced conformational changes by structural analyses of crystals.

### Factors Influencing Receptor Activity

#### Phosphorylation

A major additional process that can enhance ligand-dependent activation of receptors is phosphorylation. Steroid hormone receptors are highly phosphorylated proteins (190), and several of the phosphorylation sites have been identified on selected receptors (171–173). Certain receptor residues are phosphorylated in a ligand-dependent fashion and some are ligand-independent (171, 173, 191–193). Importantly, a significant level of phosphorylation occurs only after the activated receptor binds to DNA (174–176). Weigel et al demonstrated that this phosphorylation was carried out by a nuclear DNA-dependent protein kinase (174).

Although we know that receptors are phosphoproteins, we do not understand the precise consequences of this phosphorylation. From functional correlations, it has been proposed that receptor phosphorylation is important for gene transactivation capacity (175). Such studies have shown hormone-induced phosphorylation that precedes gene activation, both in cells (194–196), and in cell-free conditions (175). The latter appears inconsistent with one theory that predicted that phosphorylation of receptor leads to inactivation. Addi-



**Figure 18.10**

Hormone-dependent activation of the steroid/thyroid receptor family. Group A receptors represented by PR/GR and Group B by TR/RAR. See text for description. H90, heat-shock protein 90; H70, heat-shock protein 70; H56, heat-shock protein 56; and P, phosphorylation (does not represent precise number of sites).

tional evidence for a positive role for phosphorylation arises from studies on the activation of ligand-dependent receptor activity by agents that induce phosphorylation or inhibit cellular phosphatases (194, 196–198). The ability of H8, a kinase inhibitor, to dampen receptor activity in cell culture systems further supports the importance of phosphorylation in gene activation (194, 198). To date, mutational analysis has shown that a point mutation of any one of the phosphorylation sites of progesterone receptors has only a marginal effect on its transactivation potential (196). Perhaps the most dramatic effect has been demonstrated for the estrogen receptor, where a mutation in one of the N-terminal amino acids caused a significant decrease in transactivation capacity (199). It is possible that functional redundancy among phosphorylation sites makes it impossible to see a drastic effect by mutating only one of the many sites. Appropriate combinations of mutations may need to be generated to address this question. It is quite possible that still-unidentified phosphorylation sites are contributing to function. We suggest that it may be more accurate to consider intracellular liganded receptors as inherently active transcription factors whose potential simply is enhanced by phosphorylation in a cell and promoter context manner, rather than to consider phosphorylation as a “switch” for activation.

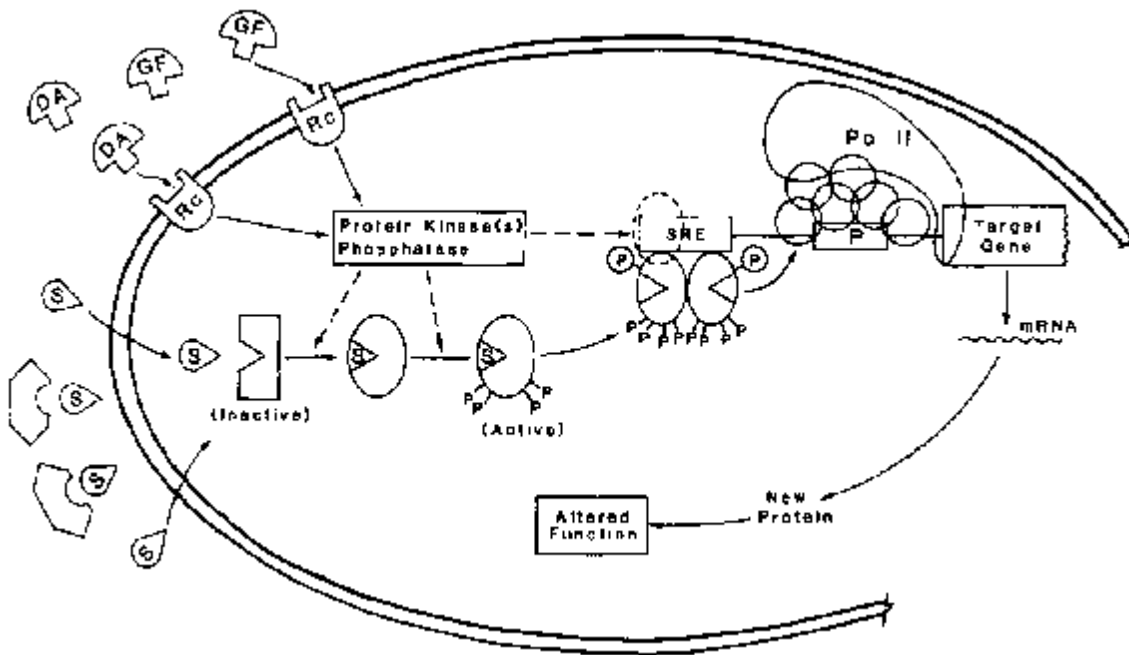
In addition to a transcriptional role, phosphorylation has been proposed to be important for receptor intracellular trafficking. Nuclear retention of GR is

inhibited by okadaic acid, a strong phosphatase inhibitor of types 1 and 2A (200). Thus, phosphorylation may be important for proper shuttling of receptor between the cytoplasm and the nucleus. Finally, phosphorylation has been proposed to be involved in receptor transformation and processing. However, RU486 has been shown to induce phosphorylation without an increase in receptor processing (201). Thus, it is unlikely that phosphorylation plays its major role in this event.

#### Ligand-Independent Activation

In addition to the putative synergistic effect of phosphorylation on ligand-dependent activation of receptor (197), several recent reports indicate that agents that stimulate intracellular phosphorylation pathways can also activate receptors in the “absence” of ligand. It was first demonstrated by Denner et al that 8-Br-cAMP and okadaic acid can activate chicken PR in the complete absence of progesterone (197). Shortly thereafter, dopamine was shown to be a biologic activator of certain steroid receptors (202, 203); also, cAMP inducers such as cholera toxin plus isobutylmethylxanthine were reported to activate native steroid receptors in a ligand-independent manner (204, 205).

To our great surprise, dopamine was shown to activate certain receptors (e.g. cPR and hER) in the absence of ligand and by events initiated at its cell membrane D1 receptors. Glucocorticoid and mineralocorticoid receptor subtypes were not activated (203). Pure antagonist ligands (e.g. ICI164, 384) prevent this



**Figure 18.11**

Dual pathways may modulate gene activation by steroid receptor superfamily members. In addition to the classical pathway, activation may be initiated at cell-surface receptors (e.g., dopamine, growth factors, etc), which results in either a presumptive phosphorylation cascade that phosphorylates receptor directly or in some "specific" cofactor needed for receptor function. See text for description. S, steroid; DA, dopamine; GF, growth factor.

crosstalk from the membrane by driving the steroid receptor into an unresponsive form (203, 205a). Finally, recent studies indicate that dopamine requires an intact PR for its effect on sexual behavior in the ventral medial nucleus of the intact rat (S Mani, J Clark, BW O'Malley, unpublished observation).

Additional recent evidence indicates that growth factors are another class of hormone that appears able to activate certain steroid receptors, particularly the human ER. In cultured cells, IGF-1, EGF, and TGF- $\alpha$  have been demonstrated to stimulate specific transcription of estrogen target genes by activating hER in a ligand-independent fashion (204, 206, 206a). There is a notable *in vivo* corollary with these experiments. EGF, acting via its membrane receptor, has been shown to cause uterine growth in ovariectomized mice that is indistinguishable from that caused by estrogen itself (205). In fact, EGF has been shown to act via its membrane receptor drive unoccupied ER to its nuclear form and turn on estrogen target genes in mice; this effect of EGF can be blocked also by administration of a pure antiestrogen (206, 206b).

This newly discovered crosstalk between membrane receptors and intracellular receptor pathways has exciting biological implications. Most certainly, it raises the question as to whether we must expand our concept of the role of intracellular receptors. As shown in figure 18.11, perhaps we should expand our model for activa-

tion of intracellular receptors to include activation from cell membrane sites. This revision may be more palatable if we consider these receptors to be transcription factors under pleiotropic regulation. Nevertheless, before we can accept the broad physiologic importance of this alternate and ligand-independent pathway for activation of receptors, we need more *in vivo* substantiation. Also, we do not understand the mechanism by which hormones whose receptors are located at the cell membrane can activate intracellular receptors; it is presumed that it might involve either phosphorylation of the receptor itself or a specific coactivator required by receptor. The fact, however, that select membrane receptor activation (e.g. growth factors, neurotransmitters, etc) can play a synergistic or additive role along with intracellular ligands to activate genes is gaining rapid acceptance. Setting aside the concept of complete ligand-independence, it appears almost certain that "cooperation" between intracellular and plasma membrane signaling pathways plays a role in the cellular physiology of certain of these receptor superfamily members.

#### Nuclear Transcription Factors

Another way to modulate receptor activity is via protein-protein interactions at the level of the nucleus. Interactions of certain steroid receptors with components of AP1 (Fos and Jun) have been shown to down-

regulate a target gene (147, 207–209). The mechanism of this modulation remains controversial. Some studies showed that interaction of the glucocorticoid receptor with AP1 results in a complex that is unable to bind to DNA (209); another report indicated that the interaction takes place while the components remain bound to DNA (210). This latter mechanism appears to be gaining favor. Others have proposed that, depending on the relative compositions of Jun and Fos, GR receptor activity either can be enhanced or inhibited (147). The confusion may derive from the use of different target genes, levels of receptors, AP1 types, and cell types. In any event, there appears to be some validity to the existence of this additional form of crosstalk between ligand-activated and phosphorylation-regulated pathways at the “nuclear” level. Again, *in vivo* studies are needed to assess the physiological importance of this phenomenon.

Finally, logic argues that intracellular receptor activity is dependent also on the cellular environment and on the local concentrations of attendant transcription factors. It is clear that overexpression of one receptor can result in the squelching of its own or another receptor's activity (211–213). Furthermore, it has been shown that the functional activity of various transactivation domains of ER varies in the context of different cell types (214). This latter result suggests that the targets for various activation domains may be distinct. Since the basic transcriptional machinery is similar for all genes, it is likely that factors other than general (TATA) transcription factors, such as coactivators, play a role in receptor activation. Using an *in vitro* reconstituted system, Shemshedini et al have identified a fraction (called TIF for transcription intermediary factor) that can eliminate the self-squelching effect of AF-1 but has no influence on basal transcription (214). Yamamoto and we have used a yeast-based genetic system to demonstrate that several factors, including SWI1, SWI2, SWI3, SIN3, and SPT6, have an effect on the transcriptional activity of different receptors (215–217). These coactivators may act to bridge receptors to the core transcription machinery or to alter chromatin structure. Overall, these reports suggest that multiple helper factors are involved in receptor transactivational activity.

McDonnell et al have used a genetic selection system to identify yeast SSN6 protein as a transcriptional repressor protein for receptors, since mutation of SSN6 results in a drastic increase in receptor activity (218). A mammalian homolog has not been identified yet. Consistent with these data, however, the silencing activity of TR can be competed by cellular over-expression of an internal fragment of the TR ligand-binding domain, indicating the existence of a soluble corepressor (A Baniahmad, SY Tsai, M-J Tsai, BW O'Malley, unpub-

lished results). Therefore, in addition to coactivators, we suggest that corepressors exist within cells. The mechanisms by which these cofactors enhance or reduce receptor activity remain to be determined, but are a subject of intense interest.

### Chromatin Structure and Receptor Action

Chromatin structure must play an important modulatory role in gene transcription *in vivo* (for reviews see 219, 220). The interaction of transcription factors with *cis*-acting elements is altered by chromatin structure, depending on whether that particular gene is in an active or repressed state. Genes located in heterochromatin are unable to bind to transacting factors and thus remain inactive. In a general sense, heterochromatin has a positive effect on transcription since it prevents the loss of limited transactors bound in unproductive complexes at inactive genes. The expression of genes in euchromatin regions also depends on the chromatin structure and the availability of *trans*-acting factors necessary for their expression. When an inducible *trans*-acting factor binds to a promoter or enhancer region of a gene, either between nucleosomes or within a nucleosome, changes are likely to be created in the local chromatin structure; general transcription factors can assemble then to form a preinitiation complex at the promoter (for review see 220). Overall, nucleosomal structure appears to act as a general repressor to restrict the availability of DNA regulatory elements.

The effect of chromatin structure on the action of steroid hormone receptors has been studied by a number of investigators (221–224). The MMTV promoter is the best-studied example of how hormone receptors alter chromatin structure to allow the expression of a target gene. In the absence of hormone, the mouse MMTV promoter contains six phased nucleosomes that prevent NF-1 and other transcription factors from binding to their *cis*-acting elements, thus preventing the expression of viral RNA (221, 222, 225, 226). The initial nucleosomal structure does not prevent activated receptors from binding to their response elements. Rather, in the presence of glucocorticoid, the GR can interact with the enhancer region of the MMTV promoter, resulting in the creation of DNase I hypersensitive sites around the GRE and in other factor-binding sites. This change is presumably caused by the removal or the modification of a nucleosome(s) within this region. Thus, a GR-induced change in the chromatin structure allows transcription factors NF-1 and others to interact with their *cis*-acting element, leading to transcription. This conclusion is supported further by Pina et al (223), who used an *in vitro* reconstitution system to demonstrate that GR can bind to *in vitro*

reconstituted chromatin with an affinity similar to its binding to protein-free DNA. In contrast, NF-1, which is able to bind to protein-free DNA with high affinity, is unable to interact with the NF-1-binding site within reconstituted chromatin. Upon binding GR or PR, the reconstituted nucleosome now becomes more accessible to exonuclease digestion, and presumably to transcription factors binding in vivo.

In addition to a general inhibitory role in factor binding, nucleosomes may promote receptor induction of transcription more directly. Schild et al (224), using the *Xenopus* vitellogenin B1 gene promoter and an estrogen-dependent in vitro transcription system, demonstrated that the formation of a nucleosome between -300 and -140 is necessary for the vitellogenin gene to be transcribed efficiently. Formation of the nucleosome is thought to create a static loop, which facilitates the interaction of an ERE located at -300 with the promoter transcriptional machinery located downstream of the nucleosome. Thus, receptor regulation of gene activity may be influenced by chromatin structure in three possible ways. It can pack unwanted genes into heterochromatin, providing a higher concentration of attendant transcription factors for induced genes. It can inhibit gene transcription by preventing binding of other required *trans*-acting factors. Finally, it can enhance receptor-factor interactions to facilitate the transcription process.

### Summary and Perspectives

In recent years, the availability of receptor cDNAs has allowed rapid progress in understanding receptor functional domains. The development of in vitro systems for assessing DNA binding and transactivation has further hastened the pace of our understanding of the detailed mechanisms by which receptors activate their target genes and of the role hormones play in this process. In this review, we have concentrated our discussion on more recent molecular advances. As is usually the case for biologic processes, however, many questions remain to be answered. An incomplete list of needed tasks and/or information includes: (a) purification of mammalian coactivators and corepressors and determination of their mechanism of action; (b) understanding of the physiological role of ligand-independent receptor activation by neurotransmitters and growth factors that regulate intracellular phosphorylation; (c) knowledge of the precise functional relevance of specific interactions of receptors with general transcription factors; (d) understanding of the relationship between silencing of the core promoter (e.g., TR) and intramolecular repression of aporeceptor (e.g., PR); (e) determination of the location mechanism of action of positive versus negative transactivational

functions within the receptor; (f) a more detailed understanding of receptor-induced changes in chromatin structure; (g) knowledge of the generality of negative HREs; (h) information on gene activational selectivity with distinct combinations of heterodimers; (i) more knowledge of receptor phosphorylation and gene regulatory capacity; and (j) determination of the precise structure of the LBD when it is coupled to ligand (agonist or antagonist).

In addition to the receptors with known ligands, there is a vast group of orphan receptors whose function and ligand are still unknown. A detailed discussion of these molecules, which represent the largest division of the superfamily, is beyond the scope of this review. At present, we are not certain if these orphans have a ligand. From their developmental distribution and timing of expression, however, it is accepted that these orphans must play important roles in tissue development and cellular physiology (227, 228). Because of the large number (>40) of orphan receptors identified to date, a great deal of experimentation is required to sort out this complex group of transcription factors. Finally, many different isoforms of both classical receptors and orphan receptors have been identified. Several isoforms of ER and a large number of forms of RAR have been shown to exist to date, most arising via alternative splicing of mRNA precursors (20, 21, 39-48, 229, 230). Variant forms of ER and PR have been demonstrated to have differential gene regulatory activities in cells in culture (41, 55, 214, 231, 232). Some isoforms of orphans are expressed in different cells, but many have overlapping expression patterns. Why do so many different receptor isoforms exist? Since this and the other questions listed above are complex, it is likely that a continuing series of breakthroughs in technology is required to achieve a complete understanding of receptor transactivation of genes. In any event, these unanswered questions should provide a good foundation for additional experimentation in this explosive field of study and will almost certainly serve as a basis for future reviews on the steroid/thyroid receptor superfamily.

### Acknowledgments

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The steroid hormones estrogen (E) and progesterone (P) regulate cellular functions in the brain that control sexual behavior in the rat (1). Facilitatory effects of P on lordosis are dependent on the prior conditioning of neural tissues with E. The cellular basis for regulatory action of E is believed to involve activation of E receptors (ER) in the ventromedial nucleus (VMN) (2) of the hypothalamus, which, in turn, act as ligand-activated transcription factors, altering the expression of a number of hypothalamic genes, including P receptor (PR) (3–9). E induction causes an increase in PR messenger RNA (mRNA) levels in the VMN of the rat hypothalamus (10), followed by enhanced PR synthesis (11). Furthermore, a strong correlation exists between the E-induced increase in PR-binding sites in the VMN and the expression of female reproductive behavior in rats (11–13). Studies using intracranial hormone implants (14–17) and localized brain lesions (18, 19) suggest that the hypothalamus, including the ventromedial nucleus (VMN), is the key site of action of both E and P for inducing lordosis. Application of inhibitors to RNA and protein synthesis to the VMN blocks E-plus P-induced lordosis (20–23), suggesting an involvement of gene expression in the control of lordosis.

The action of P has been suggested to be mediated by PR, because the P antagonist RU 38486 inhibits P-facilitated lordosis (24). This antagonist is a competitive inhibitor of hypothalamic PR binding both in vivo and in vitro. However, the role of neural PR in the facilitation of female reproductive behavior requires further clarification, because RU 38486 can competitively bind and reduce the soluble pool of PR by 50%. RU 38486 has potent antiglucocorticoid activity (25), so that the observed inhibition could be due to interference in glucocorticoid physiology. To substantiate that female sexual behavior in rodents is mediated via E-induced genomic activation of hypothalamic PR, we examined the effects of inhibition of PR gene expression in the VMN by intracerebroventricular (icv) administration of antisense oligonucleotides to PR mRNA on proceptive and receptive behaviors in rats.

## Materials and Methods

### Behavioral Testing of Animals

Ovariectomized Sprague-Dawley rats (160–180 g BW) were obtained from the supplier (Sasco or Harlan, Houston, TX). The animals were housed with a 12-h light, 12-h dark cycle and given food and water ad libitum. A week after their arrival, the animals were administered hormones and tested for sex behavior. 17 $\beta$ -Estradiol benzoate (EB; 10  $\mu$ g in sesame oil) was injected sc, followed by P sc (100  $\mu$ g in sesame oil) 48 h later. Four hours after P administration, the animals were tested for sex behavior with sexually active males housed in a 50  $\times$  45  $\times$  24-cm polystyrene arena. The proceptive and receptive behaviors of each female rat in the presence of the male were observed for 10 min, scored, and recorded. Proceptivity in the female was measured in terms of (1) hopping and darting, (2) ear wiggling, and (3) approaches to the male. Receptive behavior was measured by evaluating (1) the number of lordoses, (2) acceptance of the male by the female, (3) the number of mounts by the male, and (4) lordosis quotient. All animals that exhibited high levels of lordosis were used in the experiments. To minimize ambiguity and provide maximal quantification, the results of the experiments are expressed as the lordosis quotient (LQ), defined as the percentage of the number of complete lordosis responses (perineum elevated, all four legs extended from the initial crouch position, and head at an angle of 45° from the floor) divided by the number of mounts by the male (26).

All of the experiments described below were performed a minimum of four times each, and the observations were recorded manually on video and made in a double blind manner.

### Third Ventricle Cannulation

A stainless steel cannula (23 gauge) was implanted adjacent to the VMN of the hypothalamus into the third ventricle of the female rats using a Lab Standard stereotaxic instrument (Stoelting, Wood Dale, IL). For cannulation, the animals were anesthetized with a

combination anesthetic (0.5 ml/kg) containing ketamine (42.8 mg/ml), xylazine (8.6 mg/ml), and acepromazine (1.4 mg/ml), and the rat's head was fixed in the stereotaxic equipment. The coordinates used for the third ventricle were: antero-posterior, bregma  $-3.3$  mm; lateral, just on the midline (above superior longitudinal sinus); and dorsoventral,  $-8.5$  mm. The procedure for cannulation was similar to that described by Antunes-Rodrigues and McCann (27). Surgical tools required for cannulation and microinjections were obtained from the Bioinstrumentation Department (University of Texas Health Science System, Dallas, TX) and are the same as those described by Antunes-Rodrigues and McCann (27). Animals were allowed to recover from surgery for 1 week before use in experiments.

#### Dose Response with P

Cannulated animals were injected sc with EB (10  $\mu$ g). Forty-eight hours later, P in sesame oil at varying doses (e.g. 0.5, 5, 50, and 2  $\mu$ g) was injected into the third ventricle (icv). The proceptive and receptive behaviors of the animals were observed at various time periods after P administration and recorded.

#### Inhibition of Sexual Behavior with P Receptor Antagonists

Cannulated animals were primed with E (10  $\mu$ g). One hour before icv administration of P (2  $\mu$ g), the PR antagonists RU 38486 (2  $\mu$ g; Roussel-UCLAF, Paris, France) and ZK 98299 (2  $\mu$ g; Schering, Berlin, Germany) were injected icv into the third ventricle. Proceptive and receptive behaviors of the animals were observed and recorded as described above.

#### Administration of Sense and Antisense Oligonucleotides

Two sets of 20-mer antisense (PRAs) and sense (PRS) oligonucleotides, one set phosphorothioated and the other not, to rat P receptor A were synthesized [Synthecell (Rockville, MD) and Genosys (Conroe, TX)]. The oligonucleotides were designed such that they included the ATG site in the A form of the P receptor:

Gene sequence:

5'-TG TTG TCC CCG CTC ATG AGC 3'  
3'-AC AAC AGG GGC GAG TAC TCG-5'

mRNA:

5'-UG UUG UCC CCG CUC AUG AGC-3'

Sense oligo (PRS):

5'-TG TTG TCC CCG CTC ATG AGC-3'

Antisense oligo (PRAs)

5'-GC TCA TGA GCG GGG ACA ACA-3'

Cannulated female rats that exhibited high levels of proceptive and receptive sexual behaviors in pretests

were injected sc with EB (10  $\mu$ g). At the same time, antisense and sense phosphorothioated oligonucleotides at 4, 1.6, and 0.8 nmol were administered via the third ventricle. The oligonucleotides were administered again 24 h later via the same route; 48 h after E stimulation, P was administered icv, and sexual behavior was observed at 30 min and again at 180 min. The phosphorothioated oligonucleotides were used in these experiments because of their resistance to nuclease degradation and their effectiveness over longer durations (28, 29). Another group of rats received a nonspecific oligonucleotide containing a 20-mer sequence for directional cloning into the SP6 vector (NS-O). These oligonucleotides served as nonspecific controls. Positive controls included cannulated rats that received EB sc, followed by icv injection of P (2  $\mu$ g) 48 h later and observation of sexual behavior 30 and 180 min after P treatment. Similar experiments were performed with varying doses of the nonphosphorothioated oligonucleotides. In another set of experiments, similar treatments were given, except that a single administration of oligonucleotides was given at the time of E priming (0 h), and the second injection was excluded.

#### Cytosol P Receptor Assays

Cytosol P receptors were assayed as described previously (30). All steps were carried out at 0–4 C. The mediobasal hypothalamus was dissected out, bounded rostrally by the caudal edge of the optic chiasm and caudally by the caudal edge of the mamillary bodies. Diagonal cuts were made extending from the lateral hypothalamic fissures to the midpoint of the corpus callosum to form the lateral boundaries, and a cut below the level of the fornix formed the dorsal boundary. Tissues were homogenized in TEGT (10 mM Tris-HCl, 1.5 mM Na<sub>2</sub>EDTA, 10% glycerol, and 12 mM monothioglycerol, pH 7.4) using a Polytron tissue grinder with a PT-7 probe (Brinkmann Instruments, Westbury, NY). Homogenates were centrifuged at  $48,000 \times g$  for 30 min, and aliquots of the high speed supernatant were incubated with 0.4 nM [<sup>3</sup>H]R 5020 (final concentration; SA, 89.1; New England Nuclear Corp., Boston, MA) with or without 100 nM unlabeled P. After a 4-h incubation at 0 C, bound and free [<sup>3</sup>H]R 5020 were separated by gel filtration on  $5 \times 60$ -cm columns of Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, NJ). The protein peak was eluted into scintillation vials, scintillation fluid was added, and the samples were counted in a Packard Tri-Carb liquid scintillation spectrophotometer (Packard, Downers Grove, IL). The concentrations of cytosol protein were assayed by the method of Bradford (31), and the data are presented as femtomoles of [<sup>3</sup>H]R 5020 specifically bound per mg protein.

**Table 19.1**

Effect of icv administration of P on lordosis response of female rats

Behavior	Progesterone sc	Progesterone icv
<i>Proceptive behavior</i>		
Ear wiggle	32.3 ± 3.1	27.8 ± 2.9
Hop-dart	23.1 ± 2.9	17.8 ± 1.9
<i>Receptive behavior</i>		
Acceptance	23.8 ± 2.3	17.6 ± 1.9
No. of lordoses	23.8 ± 2.3	17.6 ± 1.9
No. of mounts	24.3 ± 2.5	26.3 ± 2.5
LQ	98.0 ± 1.2	98.6 ± 0.9

Proceptive and receptive behavior (as described in *Materials and Methods*) of each female rat in the presence of a male was scored and recorded. The results were expressed as the LQ, defined as a percentage of the number of complete lordosis responses divided by the number of mounts by the male. The responses of each female rat ( $n = 6$ ) in the presence of a male rat for 10 min was scored individually, and the mean  $\pm$  SEM were determined. Acceptance, The number of times the female allowed the male to mount. Ovariectomized female rats were primed with EB (10  $\mu$ g) in 0.1 ml sesame oil, sc, and administered 100  $\mu$ g P, sc, 48 h later. Four hours after P administration, the animals were tested for sexual behavior in the presence of sexually active males. Stainless steel cannulae were stereotactically implanted into the third cerebral ventricle of ovariectomized animals. A week after surgery the animals were primed sc with EB (10  $\mu$ g), and P (2  $\mu$ g) was microinjected into the third cerebral ventricle (ICV) and their proceptive and receptive behaviors in the presence of males were observed 30 min after P administration.

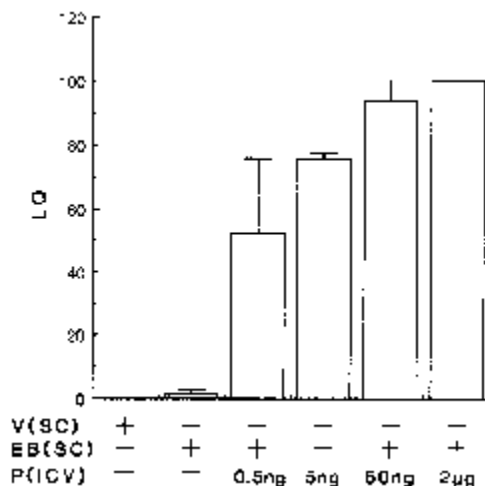
## Results

### General Behavioral Testing

EB- and P-treated (sc) animals exhibited high levels of proceptive (ear wiggling, darting, and hopping) and receptive behaviors. Mounting by the male resulted in the lordosis posture of the female and acceptance of the male by the female (receptivity). Third ventricle cannulations of ovariectomized rats did not decrease their ability to display these behaviors (table 19.1).

### Dose Response to P

Although it has been demonstrated that smaller doses of P were adequate in inducing mating behavior in hamsters when administered directly into the lateral ventricles of the brain, no dose-response studies were performed to determine the minimum dose of P required to induce lordosis (32). As we intended to administer various hormones and oligonucleotides via the third cerebral ventricle, we examined the effects of different doses of P on mating behavior. Intracerebroventricular administration of P (2  $\mu$ g) in E-primed rats resulted in their display of proceptive behavior beginning at 30–45 min and lasting for up to 3 h. At this dose, more females exhibited proceptive and receptive behaviors, and their average LQs were higher than those of animals that received lower doses (figure 19.1). Lower doses of P were characterized by irregular displays of lordosis, frequent rejection behavior, and low

**Figure 19.1**

Effects of varying doses of P on lordosis of female rats. Ovariectomized rats with indwelling stainless steel cannulas stereotactically implanted into the third cerebral ventricle were injected sc with 10  $\mu$ g EB in 0.1 ml sesame oil. Various doses of P (0.5–2  $\mu$ g) in sesame oil were administered icv 48 h later. The animals were examined for their proceptive and receptive behaviors in the presence of a male 30 min and 3 h after P administration. The results of the experiments were expressed as the LQ, defined as the percentage of the number of complete lordosis responses by the female divided by the number of mounts by the male. The control rats received 0.1 ml vehicle (sesame oil; V), and another group received EB only. The bars represent the mean LQ  $\pm$  SEM.

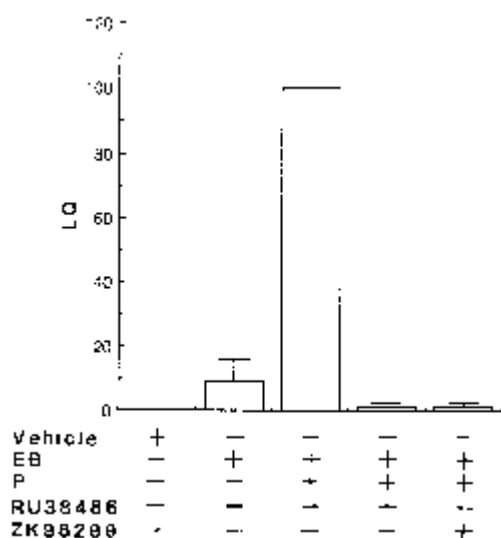
levels of hopping, darting, and ear wiggling, whereas higher doses resulted in reliable and higher levels of proceptive and receptive behaviors. In the absence of P, E-primed animals displayed minimal receptive behavior (lordosis) and no proceptive behavior. Administration of the vehicle alone (in the absence of P) did not induce proceptive and receptive behaviors. Thus, at the higher doses of P (50 ng to 2  $\mu$ g), all E-primed animals elicited reliable proceptivity and quantitative receptivity (measured and expressed as LQ).

### Inhibition of Behavioral Responses by RU 38486 and ZK 98299

Intracerebroventricular injection of the antiprogestins RU 38486 and ZK 98299 suppressed P-facilitated behavioral responses in E-primed female rats at both 30 and 180 min. Both antagonists were very effective in suppressing lordosis when administered 1 h before P (figure 19.2). Hopping, darting, and ear wiggling were also completely inhibited by the antagonists. Again, all E-primed female rats treated with P alone displayed receptive responses compared to E-primed or vehicle-administered control rats.

### Antisense and Sense Oligonucleotides and Behavior

Sexual behavior was almost completely blocked in animals treated icv with the phosphorothioated antisense



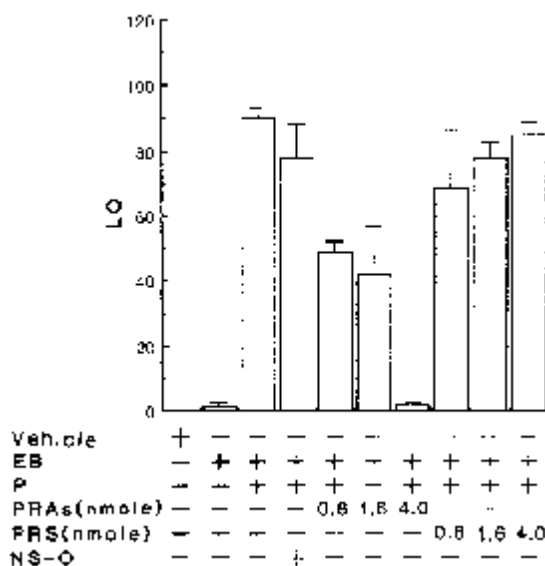
**Figure 19.2**

Inhibition of P-facilitated sexual behavior in E-primed rats by P antagonists. Ovariectomized EB-primed (10  $\mu$ g, sc) rats with stainless steel cannulas in the third cerebral ventricle were given icv injections of RU 38486 or ZK 98299 after 48 h. P (2  $\mu$ g) was microinjected icv into the third cerebral ventricle 1 h later. The animals were tested for their proceptive and receptive behaviors 30 min and also 3 h after P administration, and the receptive behavior was expressed as the LQ, as described in figure 19.1. Values presented are mean  $\pm$  SEM LQ.

oligonucleotide to rat P receptor mRNA (PRAs) at a 4-nmol dose (figure 19.3). The females adopted aggressive posture toward the males and actively kicked off the males, and lordosis was very rare. Proceptive responses (ear wiggling and hop-darting) were reduced or completely suppressed in a dose-dependent manner (figure 19.3). In contrast, rats that received the sense phosphorothioated oligonucleotide (PRS) showed high levels of proceptive and receptive behaviors. Similarly, the nonspecific oligonucleotide (NS-O; figure 19.3) had no significant effect on P-facilitated sexual behavior. Both nonphosphorothioated sense and antisense oligos had little effect on lordosis regardless of the concentration administered (data not shown). Lordosis response and receptive behavior were maximally reduced only in animals that received oligonucleotides 0 and 24 h after E priming, whereas a single administration at 0 h was only partially effective (data not shown).

#### Antisense and Sense Oligonucleotides and P Receptor Concentration in the Hypothalamus

Estradiol priming induced a 161% increase in the concentration of hypothalamic cytosol PRs, which was significantly higher ( $P < 0.001$ ) than that caused by the vehicle control treatments (figure 19.4). Infusion of antisense oligonucleotides to the PRs (PRAs; 4 nmol) caused a significant decrease (by 52.2%) in the estradiol-induced concentration of hypothalamic cytosol PRs ( $P < 0.001$ ). Pairwise multiple comparison by



**Figure 19.3**

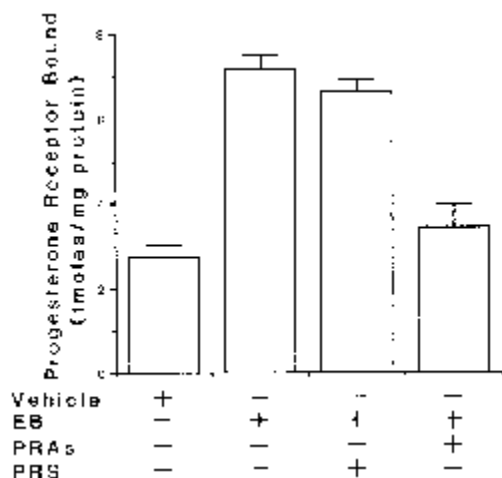
Dose response of icv administered antisense (PRAs) and sense (PRS) oligonucleotides to PR mRNA on P-facilitated lordosis behavior in EB-primed rats (10  $\mu$ g). Ovariectomized rats with indwelling cannulas in the third cerebral ventricle were injected with 0.8, 1.6, and 4 nmol of the oligonucleotides at the time of E priming. Animals were injected with the same doses of the oligonucleotides 24 h later and tested for lordosis response 30 min and 3 h after P administration. To another group of ovariectomized E-primed cannulated animals, nonspecific oligonucleotides (NS-O), consisting of a specific sequence for directional cloning into SP-6 vector, were administered in lieu of the other oligonucleotides. The animals were scored for sexual behavior, and the results are expressed as described in figure 19.1. Control animals received vehicle alone (V), E alone (EB), or P 48 h after E priming (E + P). The values presented are the mean  $\pm$  SEM LQ.

the Student-Newman-Keuls method indicated a significant effect of antisense oligonucleotides on estradiol pretreatment ( $P < 0.05$ ). However, infusion of sense oligonucleotides (PRS; 4 nmol) had no significant effect on the concentration of progesterin receptors ( $P > 0.05$ ).

#### Discussion

We have demonstrated that antisense oligonucleotides to PR mRNA administered via the third cerebral ventricle suppress P-facilitated sexual behavior in the female rat. As this treatment also reduced E-induced synthesis of PR in the hypothalamus, our results support the idea that PR is a critical component in the control of sexual behavior in the female rat.

The use of antisense RNA and DNA oligonucleotides to suppress gene activity has been successful both in vivo and in vitro (33, 34). These studies have shown that antisense oligonucleotides targeted to cellular RNA sequences can produce biological effects that have been correlated with reduced levels of RNAs or proteins. Similar effects on hormonally induced behav-



**Figure 19.4**

Mean ( $\pm$ SEM) PR concentration in cytosol from the hypothalamus of ovariectomized rats with indwelling cannula injected sc with 10  $\mu$ g EB. The antisense (PRAs) or sense (PRS) oligonucleotides to PR (4 nmol) were administered icv concurrent with EB and 24 h later. The animals were killed 48 h after EB administration, the hypothalamus was dissected and homogenized, and progestin binding assays were performed on the cytosol, as described in *Materials and Methods*. Each point is the mean of four to six independent determinations.

ior have been observed by others using direct injections of antisense oligonucleotides to PR form B into the VMN (35, 36). Although in these studies, a reduction of the receptor protein was demonstrated in T47D cells in vitro (35) and by immunohistochemistry in vivo (36), in the present study we report a reduction in cytosolic PR in the hypothalamus, as quantified by in vitro binding assays. Although it is clear that treatment of our rats with antisense oligonucleotides to PR mRNA reduced the synthesis of PR in the hypothalamus, the mechanism of action of antisense oligonucleotides is not completely clear (37). Whether the antisense inhibition in the current study is due to transcriptional attenuation of mRNA or disruption of posttranscriptional processes, such as arrested translation and/or degradation of PR mRNA, is not known at present. It is surprising that a 52.2% decrease in the E-induced cytosolic PR seen in the presence of antisense oligonucleotides completely abolished the lordosis response. However, it has been shown that similar reductions induce a hyposensitive state (30). After the termination of the period of sexual receptivity in guinea pigs, a refractory period or a period of sequential inhibition occurs, during which the animals are hyposensitive and unresponsive to P. This P-induced hyposensitivity has been correlated to reduced cytoplasmic PR and a significant decrease in the accumulation of nuclear PR in the hypothalamus (30, 38). This P-induced hyposensitivity could be overcome by administering estradiol or a large dose of P, resulting in nuclear accumulation of PR (30). Thus, it is apparent that a threshold

concentration of PR is an absolute requirement for P-facilitated lordosis, and a decrease in the concentration, but not a complete loss of the receptors, by the antisense oligonucleotides may have offset the facilitatory influence of P. The importance of threshold levels of inducible PR in the mediobasal hypothalamus-preoptic area for the expression of feminine sexual behavior has also been reported (39, 40).

Several investigators have suggested that E-induced PR is a prerequisite for P-facilitated sexual behavior (39–41). The P antagonist RU 38486 suppresses P-facilitated reproductive behavior in a dose-dependent manner when administered 1 h before P (42–45), and it abbreviates the period of sexual behavior when administered after P (45, 46). Furthermore, the antagonist appears to be a competitive inhibitor of hypothalamic-preoptic area progestin receptor binding, as demonstrated by hormone exchange assays (43, 45). Inhibition of the sexual behavior of E-primed animals by icv administration of RU 38486 1 h before P in the present study is in accordance with these reports. As RU 38486 also blocks glucocorticoid and androgen actions, it can be argued that such results are not conclusive. However, in our study we used the P antagonist ZK 98299, which is thought to have no antiglucocorticoid activity (47), to block P-induced sexual activity. These data with ZK 98299 and our results with the antisense block of PR synthesis strongly support the idea that PR plays a critical role in P-facilitated sexual behavior in the female rat.

The display of proceptive behavior in E-primed animals within 30 min after icv administration of P could be due to a direct action of P on the neuronal membranes. It has been proposed that rapid behavioral effects facilitated by P may be nongenomic and membrane receptor mediated (48). This is supported by the observations that lordosis behavior could be induced within 10 min after iv, intracerebral, or intraventricular administration of P to E-primed animals (49–52). The specific binding of P and other steroids to synaptic plasma membranes adds to the possibility that P acts to facilitate lordosis by interaction with cell surface receptors (53–56). On the other hand, these effects may involve early genomic responses that occur within minutes after hormone exposure. Such hormone-induced response patterns have been observed in the uterus (57–59). Thus, the mediation of P's action on lordosis could involve a combination of nongenomic membrane mechanisms as well as genomic actions via intracellular receptors and/or their interaction.

In summary, E acts at the level of nuclear DNA to increase the expression of the PR gene in the VMN of hypothalamus. P-facilitated lordosis behavior is mediated by P activation of its receptor. Interruption of PR gene expression using antisense oligonucleotides

inhibits P-facilitated female sexual behavior. Thus, the present data support the notion that steroid hormones initiate a cascade of events at the genomic level in the VMN of the hypothalamus to regulate the neuronal networks involved in the control of female sexual behavior in rats.

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## Introduction

The sex steroid hormones estrogen (E) and progesterone (P) control sexual behavior in the rat [1]. E treatment in ovariectomized rats is indispensable for the induction of proceptive and receptive mating behavior upon subsequent administration of P. After pretreatment for 48 h with E, administration of P is a standard protocol for initiating sexual behavior (lordosis). The primary neuroanatomical region that mediates the hormonal control of lordosis behavior is the ventromedial nucleus (VMN) of the hypothalamus [2, 3]. Treatment with E stimulates the expression of P receptor (PR) in the VMN [4, 5]. There is a strong correlation between the induction of PR binding in the VMN and the expression of female reproductive behavior in rats [6, 7]. The action of P is presumed to be a result of its binding to the PR, since the progesterone antagonist RU 38486 blocks the P-induced lordosis [8]. The role of PR is reinforced by the observation that PR antisense oligonucleotides injected into the VMN, can block P-induced lordotic behavior in the rat [1, 9].

Although the E-mediated induction of PR in the hypothalamus is well accepted, the full complement of genomic actions of E is currently unknown. To this end, we initiated a study to discover other novel genes that might be induced by estradiol.

## Experimental

### Animals

All experiments were in accordance with NIH guidelines and approved by the Institutional Animal Care and Use Committee. Studies were performed in ovariectomized Sprague–Dawley rats (Sasco, Houston, TX) weighing 180–200 g and housed three per cage in 12 h light and 12 h dark beginning at 0700 CST. Rat chow and water were provided ad libitum. Female rats were screened for positive lordosis behavior before admission to the study at 7 days after ovariectomy. Briefly, females were primed with estradiol benzoate (E, 100 µg, s.c.) followed by progesterone (P, 100 ng, s.c.) 48 h

later. Four hours after the P injection, the female was introduced for 5 min into the test arena containing a sexually proven male. Proceptive, receptive, and lordotic behaviors were observed and recorded [10]. For the experimental protocol, only females exhibiting positive lordotic behavior in response to four males were used.

### Tissue Collection

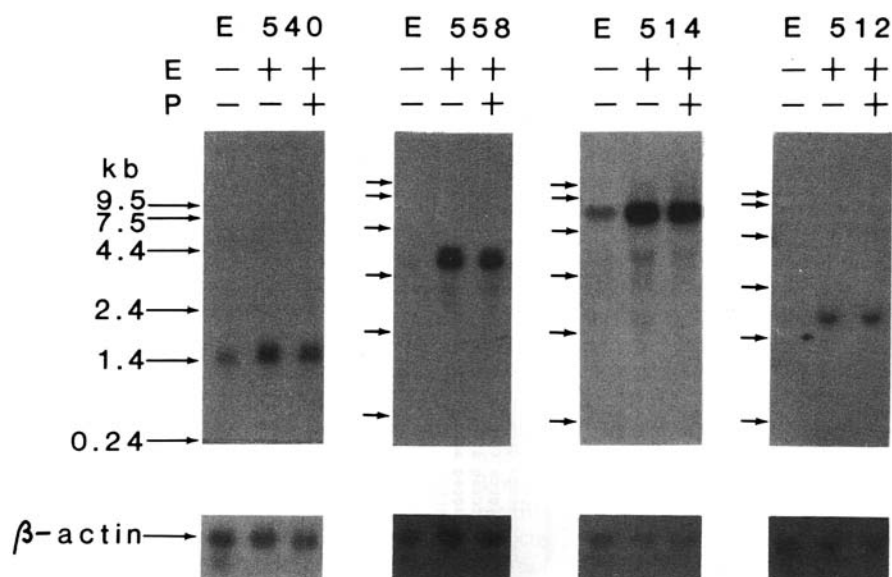
On day 21 postovariectomy, females were divided into three groups. One group received only sterile saline and was designated the no-treatment (NT) group. The other groups received either E alone or E + P as described above. Rats were decapitated; the hypothalami were rapidly exposed, dissected, and frozen for storage at –70°C until processing for RNA isolation.

### RNA Isolation and Library Construction

RNA was isolated from hypothalami using RNeasy (Qiagen, Crawfordsville, IN) as described in the manufacturer's protocol. Ten hypothalami (50 mg wt each) were pooled together from each group of animals. Each preparation yield about 400 µg of total RNA. Total RNAs were used for all Northern blot analysis. Poly A<sup>+</sup> RNAs (mRNA) were used for cDNA synthesis and library construction and were isolated from total RNA using oligo-d(T) cellulose spun column (Invitrogen Corp, San Diego, CA). A cDNA library was constructed from E + P mRNA and was cloned into the λ phage unizap XR vector (Stratagene, La Jolla, CA). Procedures for cDNA library screening were essentially the same as described previously [11, 12].

### Sequencing and Analyses

Dideoxy chain termination sequence reactions were performed using the Sequenase version 2.0 (United States Biochemicals, Cleveland, OH) and miniprep plasmid DNA as template. End sequences were obtained from each clone using T3 and/or T7 sequence primers. Sequences were matched to the Genbank using the NCBI blast program.



**Figure 20.1**

Northern blot analyses for E-induced cDNAs. Total RNAs (20  $\mu$ g) isolated from hypothalami of NT, E and E + P treated ovariectomized female rats were analyzed by formaldehyde agarose gels and transferred onto nitrocellulose filter membranes as described in Experimental. Each filter was hybridized to [ $^{32}$ P- $\alpha$ ]dCTP-labeled cDNA. Results of 4 clones: E540, E558, E514 and E512 are shown here. The bottom panels represent hybridization results of the same filters to  $\beta$ -actin cDNA probe. Molecular size markers are represented in the RNA ladder obtained from BRL. Autoradiography for  $\beta$ -actin was 30 min and 24–72 h for cDNAs.

#### cDNA Isolation and Labeling

Recombinant plasmid DNAs were isolated from 3 ml minipreps by a modified alkali lysis procedure [13]. Insert cDNAs were released by digesting with *Eco*RI and *Xho*I (Promega Corp, Madison, WI) and gel purified. DNAs were recovered from agarose gel slices by Gene-clean II (Bio 101, La Jolla, CA). Each cDNA (25–50 ng) was labeled with [ $^{32}$ P- $\alpha$ ]dCTP by random priming [14] and hybridized to Northern blots as described below.

#### Northern Blot Analyses

The Northern blot procedure of Kroczeck and Siebert [15] was employed in all RNA analyses. Briefly, 20  $\mu$ g of total RNA was applied to each lane of a 1.2% agarose gel containing 1.1% formaldehyde and 1  $\times$  MOPS (0.02 M MOPS, 0.005 M sodium acetate, 0.0001 M EDTA) buffer pH 7.0 and electrophoresed at 100 V for 3 h with no buffer recirculation. After electrophoresis, RNA was transferred to nitrocellulose membrane filters (Schleicher & Schuell, Keene, NH) by capillary action and processed as described previously [17]. RNA molecular size markers at 5  $\mu$ g per lane were used (Gibco/BRL, Bethesda, MD). Ethidium bromide at 10  $\mu$ g/ml was included in the RNA samples for better visualization of the fractionated RNA. Blots were pre-hybridized in a solution containing 5  $\times$  SSC (1  $\times$  SSC = 0.15 M NaCl, 0.015 M sodium citrate), 5  $\times$  Denhardtts (1  $\times$  Denhardtts = 0.02% ficoll, 0.02%

polyvinylpyrrolidone, and 0.02% bovine serum albumin), 0.1% sodium dodecyl sulfate (SDS) 100  $\mu$ g/ml of sheared and heat denatured salmon sperm DNA and 50% formamide at 42°C for 4 h. After prehybridization, [ $^{32}$ P- $\alpha$ ]dCTP-labeled cDNA probe was then added (1  $\times 10^6$  cpm/ml) to the same solution and hybridization was continued at 42°C for 18 h. After hybridization, filters were briefly rinsed several times with 2  $\times$  SSC followed by two 30 min washes in a solution of 0.1  $\times$  SSC and 0.1% SDS at 50°C. Filters were briefly blotted to remove excess liquid, wrapped with saran wrap and autoradiographed at -80°C between two intensifying screens.

#### Results

##### Screening and Analyses of the cDNA Library

In the course of studies in search of P-induced gene (results of which will be reported elsewhere), we screened an E + P cDNA library with probes derived from E cDNA and an E vs E + P subtracted cDNA. The E vs E + P subtraction was performed at a  $R_0t$  of 375 mol per s/l, a value much lower than the recommended 1500–3000 mol per s/l [11]. Theoretically, low  $R_0t$  hybridization removes (subtracts) abundantly expressed common sequences but not the low copy E-induced sequences. Thus, this is an enrichment procedure that may facilitate recognition of low copy induced sequences by promoting a subset of lower

abundancy mRNAs to higher abundance. Screening of the E + P cDNA library using this approach, identified 120 cDNA clones which exhibited more intense hybridization signal to the subtracted E + P cDNA probe and were selected for further analyses. DNA sequences were determined by end sequencing with T3 and T7 primers and were used to search the Genbank database for sequence homology as described in Experimental. Of the 120 clones sequenced, 16 were presently known genes; 70 clones were assumed to be unknown genes as significant sequence homology were not found in Genbank database. Among the remaining 34 clones, some were not viable and some contain mitochondrial genomic sequences and were not analyzed further. None of the identified sequences were known previously to be highly expressed genes in the brain such as actins, tubulins, myelin basic proteins, ribosomal proteins etc. Among the 86 (16 + 70) cDNAs of interest, several shared overlapping or identical sequences and therefore allowed us to focus on 70 clones for Northern blot analyses.

#### Identification of E-Induced Genes

cDNA inserts were isolated from plasmid DNA and were used to probe Northern blots of rat RNAs extracted from NT or from rats treated with E or E + P as described. We evaluated a total of 64 Northern blots (i.e., 64 cDNA clones) and found that 21 distinct mRNAs (from 28 clones) showed definitive increases in hybridization signal in the E lanes and are referred to as E-induced genes. This represented 43% (28/64) of the 64 cDNAs analyzed. Representative Northern blots and  $\beta$ -actin control blots are shown in figure 20.1. All four mRNAs showed clear induction in the E lanes. Control  $\beta$ -actin hybridization signals were uniform in all lanes and indicated that equal amounts of RNAs were loaded in each lane. The Northern blot data of all clones are summarized in table 20.1. Of the induced mRNAs, 50% were induced 3-fold or greater relative to baseline levels in ovariectomized rats. Figure 20.2 contains the deoxynucleotide sequences obtained from the 5' ends of members of the three classes of E-induced cDNA clones listed in table 20.1.

#### Discussion

The steroid hormones E and P cooperate in the induction of sexual behavior in the ovariectomized rat [1]. In order to define the molecular events involved in this complex behavioral response, it is important to understand the magnitude of the genetic response to E and to identify specific genes that may be either involved in the behavioral response or serve as gene markers

**Table 20.1**

A summary of Northern blot data: only E-induced genes are listed here

Clone No.	Identification	Estrogen Induction
VMHE505/509	Unknown	++++
VMHE518	Cytochrome bc-1 complex core P	++++
VMHE552	Unknown	++++
VMHE5103	Unknown	++++
VMHE512	Unknown	+++
VMHE514	Na <sup>+</sup> , K <sup>+</sup> ATPase B	+++
VMHE516	Unknown	+++
VMHE541	Unknown	+++
VMHE558	MRC OX45Ag deleted form	+++
VMHE569	Unknown	+++
VMHE583	Unknown	+++
VMHE593	Unknown	+++
VMHE501/543/588/5105	GMRP	+++
VMHE506/540/549	ADP/ATP transport	++
VMHE513	16 Kda H(+)-ATPase	++
VMHE536	Unknown	++
VMHE545	Unknown	++
VMHE567	Carbonic anhydrase	++
VMHE572	Unknown	++
VMHE592/594	Unknown	++
VMHE5130-3	C1-13 gene product, neuronal specific gene	++

Clones that do not match-up with sequences in the Genbank data base are listed as unknown. The level of induction is arbitrarily assigned as follows: 4+, induced from undetectable level; 3+, markedly induced (i.e. >3-fold); 2+, induced.

for the response. In the course of a study designed originally to find P-induced genes, we discovered incidentally a surprisingly large number of E-induced genes (21 mRNAs).

The mammalian brain is a highly complex organ. Earlier estimates indicated that one third of the mammalian genome is devoted exclusively to its function [17-19]. According to an estimation by Milner and Sutcliffe [20], the brain expresses approx. 30,000 distinct mRNA species of an average length of 5000 nucleotides. Kinetic [21] and clonal [20] analyses found that between 40-65% of the mass of brain mRNA was brain specific. In other words, roughly 10,000 to 20,000 of the 30,000 brain mRNAs detected in the mammalian brain are specific for that organ. This remarkable complexity means that the bulk of brain mRNAs are rare or extremely low abundant mRNAs and most likely will escape hybridization to high  $R_0t$  values.

**I. +4 INDUCED GENES**

E509  
 1 tggcgtgac tccgcacgct ggllcaattt acaatctttt caaacatat  
 51 tnnctncaaa ttccagaaat gttgttaacg gaaacagaca aaaaatatcc  
 101 acttttagttt atcaaaaata aaagaagatt taaaaaagag cacatttttc  
 151 acaaatagta ttt

E508  
 1 tggggatctc agcagctggg ttcaatttac aaactcttca aaacatattc  
 51 ctllcaaatl tacagaatgt tgttaattga acnagaGaaa acatgtccac  
 101 tttagtttat caaaaataaa agaacattta aaaaatcanc acattttctc  
 151 caaatagtat ttattttggt tgttcattta gtaatatcat aaggtttacc  
 201 gtgaacatga ttgttatatt ktaatt

E518  
 1 caagaagtca atgacagcaa gtggaaactt gggacatacg cctttctttg  
 51 acgagttata acagatgcct gtatccaggt ctcagccccg accagcaaac  
 101 acatcagngt caggtttcct acttatcaat gttatttcaa cctttttctc  
 151 aatgaagaaa aaaaactaaa agtatagtcg tccatagctc catagagcca  
 201 ataaaacatt gtatgttaat gttttctttg tactcttctc aggcagtcac  
 251 caggtattta ttaaatgctt taaaagaaac agtaataagc taatttaagtt  
 301 cacttatcac atcaattaac aattttcagaa cctgttcaaa tt

E552  
 1 tttttatgga tgaataatga atgttaattc taannactca ctccctttta  
 51 tcaacagtta taacctcagt ccattgtgct acattagcgt gctcttttgt  
 101 aataaktgtg agtatgacag ctagatgcct attggtttgt tatagaaggt  
 151 acaaatltgt tnncttttne aagtaatttc agttctagga ctgggatttc  
 201 aogtgtgttt agaatagag agaacagact gtttatatat acalatatga  
 251 acgccattca caatccttts taagg

E5103  
 1 cactcccccc agtcaggacc tccctttgct tcccagttcc agcgagcagg  
 51 atcaggtcta acgaaagctg calacctacc tgaatacagg tgcctgcccc  
 101 tgcacacacg tagctaggaa agcctgtccc aaacccagc gatccatt

**II. +3 INDUCED GENES**

E501/543/588/5105

1 gaaggttaac ggcngcngta tccgtacgtt gcaaggttag calaaacttc  
 51 gttcctcaat tagggactag aatgaatggt caaacgaggg ttaactgtct  
 101 cttactttca atcagtgaaa ttgaccttcc agtgaaaggg ctggatcttc  
 151 ccaatagagc gagagagccc tatggagcct caacttctag tccaactcat  
 201 aaaaacacaa cctaatgggc taaaacaaa taatatgaa ctaaaaattt  
 251 c

E512  
 1 ctaaaacacc cctgcacagt ctccacgtac agcagatccl taaggagact  
 51 gcccatgtct ccacacagag ctatgtcatct gccccacaga gagaggacat  
 101 agccctgagc ccccaaaa ca taggaagaaa tgagagatgc tccctgtcca  
 151 gcttgaggag gagggagaga gacaggaagt accactccct ccccaagcaa  
 201 gctgactgtt atccgtcacc gtgttttaac caacacagagc tggagaggtc  
 251 cgtgtlaacc

E514  
 1 gcatggtact ttcccttctc ttctcatttt ctgggtctg agatttcaaa  
 51 ggttaacggcc catcaacagc catltttaac acattccata gtctttccct  
 101 gtggtatcag gtcttttacka ttgtttttct ttgtttctc gggctggggg  
 151 gtgggtctgc gtgggggaac tnnedottta aatttcaa

E516  
 1 aaataattag ttggggcatt ggtttgtac agtgagctca gatcaaatag  
 51 ggaataactt gaatgtatg tctcagacca aggcacactc ccttctcat  
 101 attaaacnct ggtgtttact tctcactacg tcaacctttt taattgacag  
 151 gatlaagaaa

E541  
 1 nagaacctac gaatacctag tgacactcca laettgtac tgtagaactc  
 51 tacagacaaa gctcaagat gctgtgtgt tncactgca taaaaacggc  
 101 caatttgttc tatgttacc atctttatc agcagagact tcaatt

Figure 20.2

Sequences of E-induced hypothalamus cDNAs. Only the sequences derived from the T3 primer (5' end) are listed.

E558 1 ggaatgcatt tatctttata aaaaagagtg atataacaa cactagata  
 51 alatagccac atgttttttg taaaaggat gcatctatct ttataaaca  
 101 gagtgatata aacacatta gcatgcata gtcacataat ttgttaaaag  
 151 gaatgcattt atcttcaca aaaggagtga tat

E569 1 ctttagcttc cagaacagg tatgggatag atctttatct cagttcatgg  
 51 ccaatnccc taactcaatt ccagcttga gtcctcttgc atttcad

E593 1 atttcacat gtcctctaga agtcattaa agagagctgc agtcttcag  
 51 accagctctt cggacgggtg attatcctca ggttcactcg gggacatta  
 101 agagagctgg aatggtgag ccaaac

E563 Not Determined

### III. +2 INDUCED GENES

E506/540/549 1 caatgkcatc agatacttcc caccacaggc tctcaacttt gcttcaaaq  
 51 ataaatacaa cagatcttt ttgggtggtg tggacaagag gacccagttt  
 101 tncgggtact ttgcaggga cctggcctca ggt

E513 1 ctggagacaa gctctgaga actctctacc ctcccccggc tgttcaaggc  
 51 tggggggcag gtggtctcat ccaaggagtc tggcacacag gacccacaa  
 101 aggcacacag ctactaatga tcactctaga gttgggagac aggggtgt

E517 1 gatgagcaga gacacctgac tggcatttca tctgggagcc aggcgacttc  
 51 gcaaggtgta tccaaacag agctccatct gcggggactg taactgtggg  
 101 tcaactccag atctctgact gctcttggcc gctgactgaa gaggagacnn  
 151 tgcagagag agtgcctttt ccatctggac acaaacaaq ggccttttgt  
 201 aggattttct

E536 1 aaatgcagct ctgcgtaaag cgtcggcttg ggggctagag ggglgggct  
 51 taagttcttc ttggatttt aatgaaagat cgtatcggga gcagttttct  
 101 tctgccttc accgcacttc tccaatccgt atgacacac atccatttca  
 151 gagcatttca gagctgcttg gcttctgttg aagagttaaa ggaacggg

E515 1 aaattggaag tgaagacatg tattgcccac gggattaaaa aaatataaaa  
 51 gaaaagaaaa gaaaaaatca gaggatgltt cccatcagg agggaggggg  
 101 cggggggaat ccaaatagta tttctgtggg gaattatata atataccttc  
 151 agtcacattg accagtcagt cctggatttt agagat

E567 1 gaattctnta cctggatagt gctcaaggaa cccattactg tcaagcgtga  
 51 gcaagatgta ccttccgta aactgaactt aattcggagc agganctga  
 101 agactgatg gt

E572 1 tgtacaaaa gaaactcta gacaatttta agctagagc cgttcaacgc  
 51 tgcctttagt cacttaaat ctctcccaa aatacttcc taatatata  
 101 aactacacaa ccttgcatt ttaagtctgg

E592/594 1 cgcagagggc tctggtctgg agctggcca aagaagttcc tagcctttcc  
 51 ctttcccta aactgcata gggagaaac ctatcgcta gcttggcttt  
 101 cactgaggt tttctgagga aggggacttg gataagagct ctgtcagta  
 151 gtaagtaga caagaaact tattaatcca gtt

E5130-3 1 cttgctttac aagacgccac atccactatc atagaagaa ttacaaact  
 51 tcatgacac accctaatca ttgtatctct catcagctcc ctactacttt  
 101 atactatttc accactacta ccaacaaac taacacacac aagcacata

Figure 20.2  
(continued)

Therefore, the strategy for this study was to use a combination of low  $R_0t$  hybridization and differential screening to help identify induced genes. The results using differential library screening to detect P-induced genes will be presented elsewhere. Theoretically, low  $R_0t$  hybridization removes abundantly expressed common mRNAs but not the low copy induced sequences. Thus, this is an enrichment procedure that may facilitate recognition of low copy induced sequences by promoting the lower abundance mRNAs to a level of higher abundance. This approach was employed to detect E-induced mRNAs. The fortuitous identification

of a large number of E-induced genes appears to be the result of this enrichment effect.

Gonadal steroids, especially E, regulate many brain functions and E-concentrating cells are abundant throughout the brain [22–24]. Estrogen has been shown to up-regulate a variety of genes in the hypothalamus, including regulatory proteins such as the PR [4] and c-fos [25, 26], rate-limiting enzymes such as tyrosine hydroxylase [27], and neurotransmitter molecules such as neurotensin/neuromedin N [28], somatostatin [29], gonadotropin-releasing hormone (proGnRH-GAP [30]), preproenkephalin [31], and

oxytocin receptor [32, 33]. Ironically, these cDNAs were not among the clones identified in hypothalamic tissue samples in the present study. Methodological differences in our approach may explain the different findings. Virtually all previous studies demonstrated mRNA regulation using preselected and targeted cDNAs or antibodies. Also, variation in experimental protocols such as surgery, time of tissue collection, and the amount of hormone treatment may have contributed to differences in the detected gene induction pattern. Finally, a large proportion of the above studies were performed using biochemical methods such as binding [32] and immunocytochemistry [26] or by in situ hybridization analyses [4, 27–29, 31]. None of these methods are ideal for measuring relative levels of mRNA expression in the whole hypothalamus.

Other studies that employed more quantitative methods such as Northern [29], dot-blot [31] and RNA protection assays [28], were performed on microdissected subregions of the hypothalamus. Even then, the magnitude of mRNA induction was found to be very small in many instances. For example, an E-induced increase of somatostatin mRNA in the preoptic area and the mediobasal hypothalamus, was detected by in situ hybridization studies [35]. However, Northern blot analysis of whole hypothalamus RNA showed only a 0.5-fold E-induced increase in somatostatin mRNA level [29] and required large amount of mRNA (10  $\mu$ g, poly A+) for hybridization signal. Thus, mRNA induction in specific cell groups when readily revealed by in situ hybridization, may not be detected readily by Northern blot analyses due to tissue dilution effects. Consequently, some E-induced mRNAs may have escaped detection due to a tissue dilution effect and remain as very low abundant mRNAs even after enrichment by subtraction.

Morphologically, 30% of neurons in the VMN respond to E by showing an increase in size of cell nuclei, nucleoli and cell somatae [36]; an abundance of stacked RER and polyribosomes indicative of active metabolism are also observed after E administration [36, 40]. Gonadal steroids may induce synaptic remodeling in the adult rodent hypothalamus, since the number of synapses in the arcuate nucleus [35–37] and VMN [38] have been shown to increase with E treatment. A number of the E-induced mRNAs shown in table 20.1 encode metabolic enzymes, and are also moderately abundant. Therefore, some of the E-induced genes in this report may be the product of a broad targeting effect of E, thus, providing some molecular basis for the observed morphological changes in the hypothalamus.

Although the present sequence information is limited to 5' plus 3'-end sequences, it is interesting to note that the vast majority of clones reported herein do not represent known sequences in the Genbank. Considerable

interest has been generated recently for obtaining expressed sequence tags, especially from the brain [40, 41]. This study represents the largest number of E-induced genes ever described for a single organ. In the rat uterus, which undergoes a marked growth response to E, only a few specific marker genes have been shown to be under estrogenic regulation. Our finding of a more global response to estrogen, casts a new light on the magnitude and complexity of the effect of this hormone in the rat hypothalamus. Finally, the newly identified cDNAs may serve as markers for E-induced central nervous system behavior and deserve further investigation into their physiologic roles. Some of these gene products could provide additional insights into the molecular basis of steroid hormone action in sexual behavior, and also may be of use in studies of estrogenic responses in other organs and disease states.

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The actions of adrenal and gonadal steroids, thyroid hormone, and vitamin D are mediated by receptors encoded by a family of related genes. Mutations of glucocorticoid, androgen, thyroid hormone, and vitamin D receptors leading to syndromes of hormone resistance have been reported (1, 2, 3, 4). It has been thought that mutation of the estrogen-receptor gene would be lethal, affecting embryo implantation in particular (5). Recent insertional disruption of the mouse estrogen-receptor gene (6) and two case reports (7, 8), however, raise questions about the validity of the lethality hypothesis and reveal intriguing phenotypes. In mice with disrupted estrogen-receptor genes, both sexes are viable. The affected female mice have hypoplastic breasts and uteri, hyperemic cystic ovaries without corpora lutea, infertility, and decreased skeletal mineralization; the male mice have decreased skeletal mineralization and low sperm counts.

The first case report (7) involving decreased estrogen synthesis described a female patient with pseudohermaphroditism due to placental aromatase deficiency, suggesting that, at least beginning late in the first trimester, excess androgen with low or absent estrogen in the female fetus is compatible with life. The second case report (8) described a karyotypically female patient with pseudohermaphroditism caused by a null mutation in the aromatase cytochrome P-450 gene. At puberty progressive virilization without breast development or growth acceleration was noted. Estrogen treatment resulted in normal breast development, a pubertal growth spurt, and menarche, suggesting that androgen is relatively ineffective in stimulating pubertal growth.

In this report, we describe a man with estrogen resistance who had osteoporosis, unfused epiphyses, and continuing linear growth in adulthood. He also had elevated serum estrogen concentrations, abnormal gonadotropin secretion, and no target-tissue responses to estrogen therapy. Analysis of the estrogen-receptor gene revealed a change in a single base pair in the second exon, generating a premature stop codon. These findings indicate that estrogen-receptor mutations need

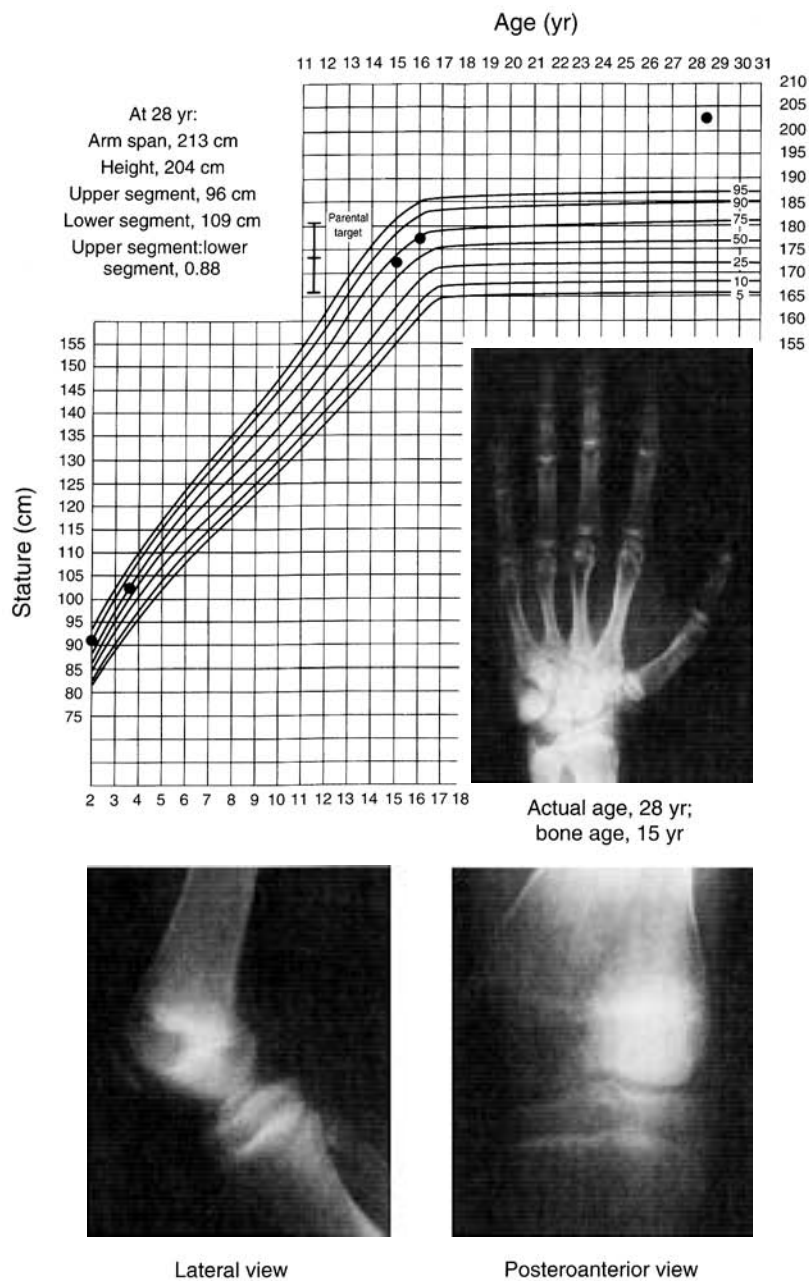
not be lethal and that estrogen is important in the male, as it is in the female, for normal skeletal growth and development.

### Case Report

A 28-year-old white man presented to an orthopedic surgeon with tall stature and a four-to-five-year history of progressive genu valgum. Because radiologic evaluation revealed unfused epiphyses, he was referred for further evaluation. His birth weight and early growth and development were within normal limits, and his stature was in the average range. He first noted pubic and axillary hair at 12 to 13 years of age and started shaving regularly at age 17 to 18 years of age but had no recollection of associated growth acceleration. His height at the age of 16 was approximately 178 cm (70 in.) according to information provided on his driver's license (figure 21.1). His legs and feet continued to grow slowly after adolescence.

Review of the family history revealed four sisters who were average in stature (160 to 165 cm [63 to 65 in.]). His mother was 162 cm (64 in.) tall, and his father was 180 cm (71 in.). His mother was given a diagnosis of non-insulin-dependent diabetes mellitus at the age of 45 years. A paternal uncle had colon cancer, two maternal cousins had insulin-dependent diabetes mellitus (one of whom was given the diagnosis in infancy), and a niece had XO/XY mixed gonadal dysgenesis.

The patient had a history of Osgood-Schlatter disease, which was diagnosed when he was 20 years of age and was treated with rest. At the age of 24, a meniscal tear in the right knee required arthroscopic surgery and the results of a glucose-tolerance test were reportedly slightly abnormal. There was no history of polyuria, polydipsia, blurred vision, or nocturia. The patient did not recall any change in facial structure, thickening or oiliness of the skin, excessive diaphoresis, skin tags, or changes in his voice. He did remember noticing increased pigment in the skin of each axilla starting at the age of 23. Though unmarried, he reported no history of gender-identity disorder. He

**Figure 21.1**

Growth chart of a 28-year-old man with estrogen resistance and radiographs of the left hand and wrist and left knee. Anthropometric measurements made at the age of 28 years are also shown.

**Table 21.1**

Biochemical measurements before and after four and six months of transdermal ethinyl estradiol therapy in a man with estrogen resistance

Variable*	Base Line	4 Mo	6 Mo	Normal Range
<i>Serum</i>				
Alkaline phosphatase (U/liter)	205	—	—	35–95 (adulthood) 50–375 (puberty)
Glycosylated hemoglobin (%)	9.5	—	—	4.4–8.8
Calcium (mg/dl)	8.8	—	—	8.8–10.8
Phosphorus (mg/dl)	3.4	—	—	2.5–4.7
Magnesium (mg/dl)	1.9	—	—	1.8–2.4
Parathyroid hormone (pg/ml)	16	—	—	10–65
1,25-Dihydroxyvitamin D (pg/ml)	31	—	—	17–44
25-Hydroxyvitamin D (ng/ml)	15	—	—	11–68
Growth hormone (ng/ml)	<0.6	—	—	≤10
Insulin-like growth factor I (ng/ml)	528	—	—	123–465 (adulthood) 395–776 (puberty)
Follicle-stimulating hormone (mIU/ml)	33	30	30	2–15
Luteinizing hormone (mIU/ml)	37	37	34	2–20
Testosterone (ng/dl)	445	—	—	265–800
Free testosterone (ng/dl)	16	—	—	5–21
Dihydrotestosterone (ng/dl)	24	—	—	25–75
Estrone (pg/ml)	145	—	—	16–65
Estradiol (pg/ml)	119	272	250	10–50
Free estradiol (pg/ml)	0.6	6.6	6.4	0.3–0.9
Sex hormone-binding globulin (nmol/liter)	6.0	10.0	7.0	0.4–4.8
Thyroxine-binding globulin (mg/dl)	2.8	2.7	2.5	1.7–3.6
Cortisol-binding globulin (mg/liter)	24	25	27	19–45
Prolactin (ng/ml)	9.7	6.6	5.9	3–15
Osteocalcin (ng/ml)	18.7	21.6	19.4	3–13
Bone-specific alkaline phosphatase (ng/ml)	34.2	35.9	33.3	4.3–19.0
<i>Urine</i>				
Pyridinium (nmol/mmol creatinine)	110	130	116	20–61
D-Pyridinium (nmol/mmol creatinine)	32	35	34	4–9
Telopeptide (nmol BCE/mmol creatinine) <sup>†</sup>	248	241	239	23–110

\*To convert values for calcium to millimoles per liter, multiply by 0.2495; to convert values for phosphorus to millimoles per liter, multiply by 0.3229; to convert values for magnesium to millimoles per liter, multiply by 0.4114; to convert values for 1,25-dihydroxyvitamin D to picomoles per liter, multiply by 2.4; to convert values for 25-hydroxyvitamin D to nanomoles per liter, multiply by 2.496; to convert values for testosterone, free testosterone, and dihydrotestosterone to nanomoles per liter, multiply by 0.03467; to convert values for estrone to picomoles per liter, multiply by 3.699; to convert values for estradiol and free estradiol to picomoles per liter, multiply by 3.671; and to convert values for thyroxine-binding globulin to nanomoles per liter, multiply by 12.87.

<sup>†</sup>BCE denotes bone-collagen equivalent.

indicated strong heterosexual interests and had normal functioning, including morning erections and nocturnal emissions.

Physical examination revealed a tall, healthy-appearing man with no acromegaloïd features but with obvious genu valgum. His height was 204 cm (80.3 in.) (>95th percentile), weight 127 kg (280 lb), heart rate 80 per minute, and blood pressure 140/85 mm Hg. The upper segment of his body was 96 cm (37.7 in.), and his lower segment was 109 cm (42.9 in.), yielding a ratio of the upper segment to the lower segment of 0.88 (average for men, 0.96). His left middle finger measured 10 cm (3.9 in.) (97th percentile, 9 cm), left hand 23 cm (8.9 in.) (97th percentile, 19.5 cm), and foot 33 cm (13 in.) (97th percentile, 29 cm). He had an arm span of 213 cm (83.8 in.). There was bilateral axillary acanthosis nigricans and a 0.5-cm skin tag in the left axilla. The patient had a full beard with early temporal

hair loss. There was no thyroid enlargement or gynecomastia. The results of cardiovascular, respiratory, and abdominal examinations were normal. The patient had normal male genitalia with bilateral descended testes, each with a volume of 20 to 25 ml, and a normal-sized prostate gland.

Radiography of his left wrist and hand revealed a bone age of 15 years (9) (figure 21.1). Knee films revealed open epiphyses (figure 21.1); a review of radiographs from previous orthopedic evaluations demonstrated minimal evidence of epiphyseal maturation over a 10-year period and demineralized bones. The density of the lumbar spine, measured by dual-energy x-ray absorptiometry (Hologic, Waltham, Mass.), was 0.745 g per square centimeter (3.1 SD below the mean for age-matched normal women and more than 2 SD below the mean for 15-year-old boys [the patient's bone age]). The karyotype was 46,XY.

Semen analysis revealed a sperm density of 25 million per milliliter (normal, >20 million per milliliter), with a viability of 18 percent (normal, >50 percent). The results of initial laboratory tests are shown in table 21.1. The serum testosterone concentration was normal, and estradiol, estrone, follicle-stimulating hormone, and luteinizing hormone concentrations were high. A five-hour oral glucose-tolerance test (75 g) revealed a fasting blood glucose concentration of 135 mg per deciliter (7.6 mmol per liter), a peak response of 224 mg per deciliter (12.5 mmol per liter) at three hours, and a concentration of 163 mg per deciliter (9.1 mmol per liter) at five hours. The respective serum insulin values were 50 micro U per milliliter (300 pmol per liter), 114 micro U per milliliter (684 pmol per liter), and 93 micro U per milliliter (558 pmol per liter).

On the basis of the hypothesis that primary estrogen resistance might explain the elevated serum estrogen and abnormal serum gonadotropin concentrations, failure of epiphyseal fusion, and possibly insulin resistance, the patient was treated with high-dose transdermal ethinyl estradiol (Estraderm patch system, Ciba, Summit, N.J.) for six months. The starting dose was 2100- $\mu$ g patches per week, with 100- $\mu$ g increments each week until a maintenance dose of 14100- $\mu$ g patches per week was reached. The dose was based on clinical experience with men treated with high doses of estrogen for either prostate cancer or transsexual conversion (10, 11). This protocol was approved by the Cincinnati Children's Hospital institutional review board, and the patient gave informed consent.

The serum hormone and metabolic measurements before and after four and six months of estrogen therapy are shown in table 21.1. (All serial tests in table 21.1 were performed at the Nichols Institute, San Juan Capistrano, Calif., and for each assay, results were analyzed simultaneously for purposes of comparison).

During estrogen therapy, the patient had no nausea, fluid retention, hypertension, unusual headaches, weight gain, gynecomastia, impotence, or mood alterations. In addition, there was no significant increase in the serum concentration of any estrogen-dependent protein (sex hormone-binding globulin, thyroxine-binding globulin, cortisol-binding globulin, or prolactin) or change in serum gonadotropin concentrations (table 21.1). The results of tests of bone turnover were all consistent with active bone demineralization and did not decrease. Finally, total bone mineral density and bone age did not change during estrogen administration.

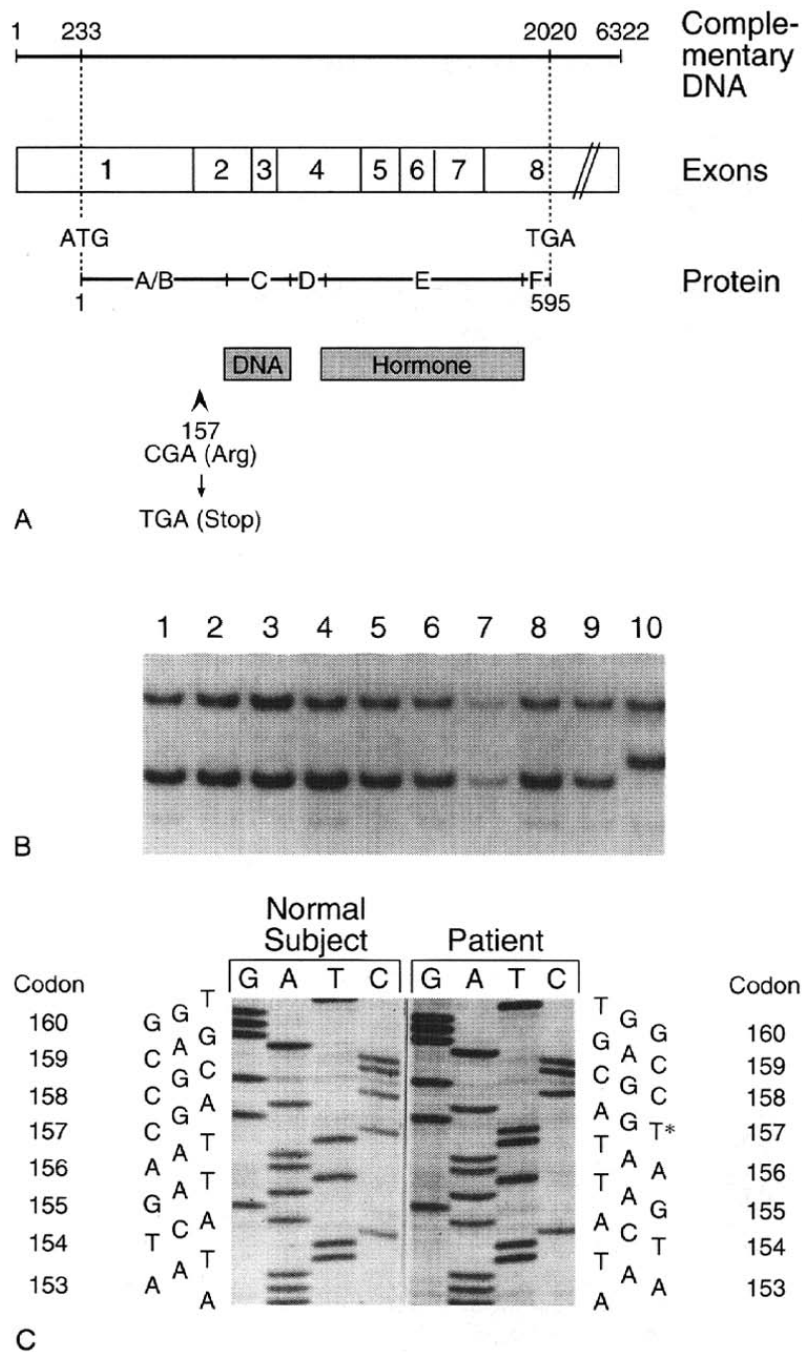
Because of the patient's resistance to estrogen, peripheral-blood lymphocyte DNA was obtained to study his estrogen-receptor gene by analysis of single-strand-conformation polymorphisms (12). Exons 1 through 8 (13) were independently amplified by the

polymerase chain reaction (PCR) and subjected to single-strand-conformation analysis with DNA from several normal subjects as a control. All exons were wild type, with the exception of exon 2, which had a variant banding pattern suggestive of a homozygous mutation (figure 21.2B). Direct sequencing of the exon 2 product revealed the substitution of thymine for cytosine at codon 157 (figure 21.2C), resulting in the replacement of an arginine codon (CGA) with a premature stop codon (TGA). The translated protein would therefore be severely truncated, lacking the DNA-binding and hormone-binding domains (figure 21.2A), and expected to be functionally inert. Because the mutation was homozygous, the patient's parents were interviewed to obtain a more detailed family history. The family pedigree (figure 21.3) demonstrates that his parents were second cousins. On the basis of slot blot analyses performed with wild-type and mutant oligonucleotide probes (figure 21.4A) and direct sequence analysis (figure 21.4B), each parent, as well as three of the patient's four sisters, proved to be heterozygous for the mutation, a finding consistent with autosomal recessive inheritance.

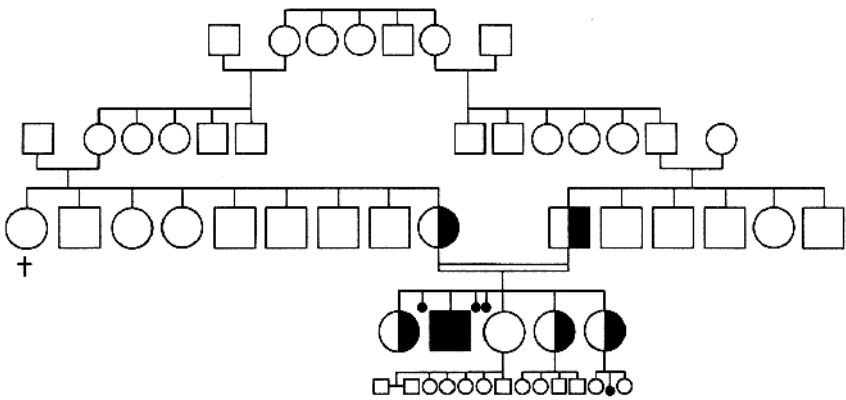
## Methods

The coding region of the estrogen-receptor gene was amplified by PCR. Primer sequences for the eight coding exons of the gene were designed on the basis of information on the exon-intron junction sequence (13). The forward primer sequence for exon 2 was 5'CCCAGGCCAAATTCAGATAA3', and the reverse primer sequence was 5'CGTTTTCAACACACTATTAC3'. PCR was carried out with 35 cycles consisting of one minute at 94 °C, one minute at 55 °C, and one minute at 72 °C. The reaction mixtures were heated at 94 °C for 90 seconds before the first cycle and at 72 °C for 7 minutes after the last cycle. After amplification, a 4-microl aliquot of the product was diluted with denaturing buffer, heated at 95 °C for five minutes, and cooled on ice for five minutes; 3 to 4 microl of this solution was used for electrophoresis.

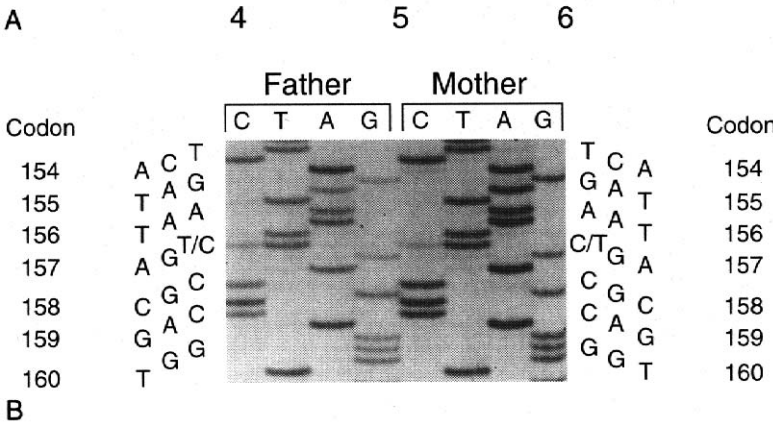
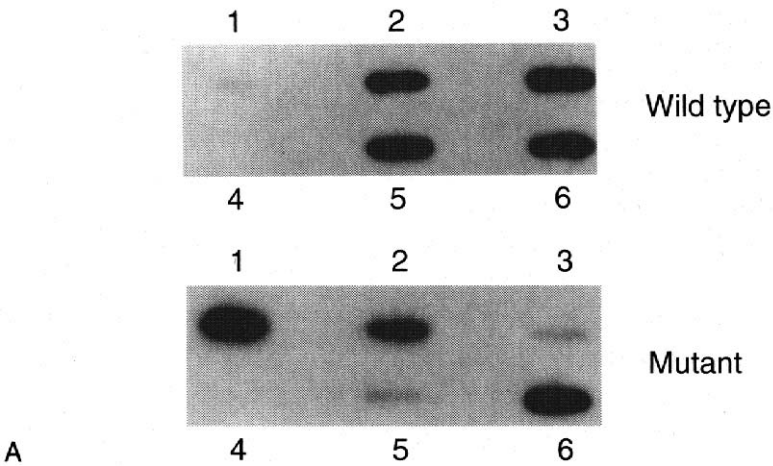
The gels used for single-strand-conformation analysis consisted of 0.5 $\times$  MDE solution (AT Biochem, Malvern, Pa.) and 0.6 $\times$  TBE buffer (89 mM Tris, 89 mM borate, and 2 mM EDTA), and they were run in 0.6 $\times$  TBE buffer at 8 W for 16 hours at room temperature. For sequence analysis, both strands of the purified exon 2 products obtained by PCR were subjected to cycle sequencing with a double-stranded DNA Cycle Sequencing System (Life Technologies, Gaithersburg, Md.) as specified by the manufacturer, except that [ $\gamma$ -<sup>33</sup>P]ATP was used for primer labeling. Sequencing reaction samples were run on a 6 percent polyacrylamide gel containing 8.3 M urea at 70 W for two to

**Figure 21.2**

Mutation of the estrogen-receptor gene. *Panel A* shows the location of the codon 157 mutation in the estrogen-receptor protein. The mutation occurs in the A/B domain that is upstream of both the DNA-binding and hormone-binding domains and would be expected to yield a severely truncated protein with no functional activity. *Panel B* shows the results of single-strand-conformation analysis of exon 2 of the estrogen-receptor gene. A homozygous sequence variant is present in the patient's DNA (lane 10) but not in DNA from nine normal subjects (lanes 1 through 9). In *Panel C*, sequence analysis of the exon 2 PCR product revealed that the patient had thymine substituted for cytosine at codon 157 (indicated by the asterisk).



**Figure 21.3**  
Pedigree of a man with estrogen resistance demonstrating consanguinity (double lines). Sequence analysis of the estrogen-receptor gene was performed only in the proband, his parents, and his sisters. Squares denote male family members, circles female family members, the solid square the homozygous proband, half-solid symbols family members who were heterozygous for the estrogen-receptor mutation, the cross a stillbirth, and small circles spontaneous abortions.



**Figure 21.4**  
Parental origin of the mutation of the estrogen-receptor gene. *Panel A* shows the results of DNA slot blotting after hybridization with radiolabeled oligonucleotide probes specific for the wild-type or mutant sequence of exon 2. The filter contained PCR-amplified exon 2 from DNA of the patient (slot 1), his father (slot 2), his mother (slot 6), and two normal subjects (slots 3 and 5). For slot blot analysis of parental DNA, the exon 2 products obtained by PCR were blotted and hybridized to radiolabeled oligonucleotide probes as described (14). The oligonucleotide sequences were as follows: 5'TTCAGATAATCGACGCCAGGG3' (wild type) and 5'TTCAGATAATTGACGCCAGGG3' (mutant). In *Panel B*, sequence analysis of the exon 2 PCR product indicated constitutional heterozygosity for the codon 157 mutation in both parents.

four hours at room temperature and processed for autoradiography according to standard procedures.

## Discussion

The findings in this man with a naturally occurring disruptive mutation of the estrogen-receptor gene demonstrate that mutations in this gene need not be lethal. The major phenotypic manifestations of estrogen resistance that he demonstrated were tall stature with evidence of continued slow linear growth, markedly delayed skeletal maturation, and osteoporosis. These abnormalities provide compelling evidence of the critical part played by estrogen in bone development and mineralization during puberty not only in girls but also in boys.

The pubertal growth spurt and epiphyseal maturation are considered to be induced primarily by the actions of sex steroids, estrogen in the female and androgen in the male. The close association of sex steroids and advancement of bone age is well demonstrated in precocious puberty, which is characterized by a premature increase in sex-steroid secretion, increased height velocity, accelerated epiphyseal maturation, and reduced final adult height (15). Despite the normal timing of pubertal onset and normal serum androgen concentrations, this adult with estrogen resistance had a bone age of 15 years and a slow continued increase in height during his third decade. His presentation is similar to that of a genetic female with pseudohermaphroditism caused by an aromatase-gene defect in whom androgen was present in the absence of circulating estrogen, rather than estrogen resistance (8, 16). Despite virilization at puberty, the patient's bone age was delayed relative to her chronologic age, and she had no growth spurt (16). However, unlike the results in this man with estrogen insensitivity, her treatment with estrogen resulted in growth acceleration, advancement of bone age, and breast development. Finally, our patient's phenotype is consistent with that associated with two other conditions, testotoxicosis and androgen insensitivity. In testotoxicosis, in which there is autonomous production of androgen from the testes, therapy with an antiandrogen alone is not sufficient to slow skeletal growth to a prepubertal rate; this is achieved by the addition of an aromatase inhibitor (17). Patients with complete androgen insensitivity have a pubertal growth spurt that is normal for a genetic female in both magnitude and timing (18). This man's phenotype confirms what these earlier clinical observations suggested—namely, that estrogen has a critical role in pubertal growth and epiphyseal maturation in both sexes.

Although the importance of estrogen deficiency in the pathogenesis of osteoporosis in postmenopausal

women is well known, many clinical observations have supported the idea that androgen is important for the maintenance of bone mass in men. Men with hypogonadism have osteoporosis (19, 20); decreased serum testosterone concentrations in elderly men are a risk factor for fractures (21); men with a history of constitutional delay of puberty have decreased bone density as adults (22); and androgenic steroids increase bone mass (23). This man with estrogen resistance had a severely undermineralized skeleton with biochemical evidence of increased bone resorption (24, 25) despite normal serum androgen concentrations. These observations indicate that androgen alone is not sufficient to promote skeletal maturation and retain bone mass and that estrogen has a pivotal role in the mineralization of the skeleton in males as well as females.

The elevated serum estrogen concentrations in this man suggest a compensatory increase in aromatase activity in response to estrogen resistance, and increased aromatase activity could account for the normal concentrations of androgen despite increased secretion of luteinizing hormone. In men, multiple tissues are involved in the aromatization of androgen to form estrogen, including the testes, liver, skin, and adipose tissue (26, 27). Testicular estrogen secretion is stimulated by luteinizing hormone, making the testis the most likely source of the elevated serum estrogen concentrations in this man. His elevated gonadotropin secretion suggests that estrogen plays a part in the regulation of gonadotropin secretion in men.

The relation of insulin resistance, glucose intolerance, and acanthosis nigricans to estrogen resistance in the patient is intriguing. Isolated increases in estrogen improve glucose tolerance by enhancing either target-tissue responsiveness to insulin or insulin secretion (28, 29, 30). Acanthosis nigricans is a cutaneous marker of insulin resistance, especially when insulin resistance is associated with relative hyperandrogenism (31). In this man, loss of estrogen effect or an altered balance of androgen and estrogen action may well account for diminished insulin sensitivity, glucose intolerance, and acanthosis nigricans. The elevated serum concentration of sex hormone-binding globulin, an estrogen-dependent protein, is unexplained.

It is possible that mutations causing milder estrogen resistance exist, and that compensatory hyperestrogenemia can overcome the resistance and result in a normal phenotype. The absence of lethality and the rather striking phenotype in either sex suggest that previous cases would eventually have been appropriately diagnosed. It is possible that heterozygous women may have impaired fertility, thus reducing the incidence of the mutation in the population and decreasing the prevalence of homozygous cases. Notably, the patient's mother had three spontaneous abortions. Regardless,

this patient's diagnosis suggests that there are likely to be other patients with phenotypic presentations of variable severity. Estrogen-receptor defects should be included in the differential diagnosis of such apparently disparate entities as tall stature, unfused epiphyses, osteoporosis, abnormal gonadotropin secretion, and infertility.

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Investigators interested in reproductive behavior have demonstrated that one role of the gonadal hormones in adult male and female mammals is to bring to expression the patterns of behavior previously organized or determined by genetical and experiential factors (1, 2, 3, 4, 5). The hypothesis that these hormones have an organizing action in the sense of patterning the responses an individual gives to such substances has long been rejected (6, 7, 8, 9, 10). As far as the adult is concerned, this conclusion seems well founded. Female hormone, instead of feminizing castrated male rats as Kun (11) claimed, increased their activity as males (6). Male and female guinea pigs gonadectomized the day of birth, and a female rat with a congenital absence of the ovaries, displayed normal patterns of behavior when injected with the appropriate hormones as adults (3, 9, 12).

Unexplored since the studies of Dantchakoff (13, 14, 15), Raynaud (16) and Wilson, Young and Hamilton (17), is the possibility that androgens or estrogens reaching animals during the prenatal period might have an organizing action that would be reflected by the character of adult sexual behavior. If the existence of such an action were revealed, it would 1) extend our knowledge of the role of the gonadal hormones in the regulation of sexual behavior by providing information bearing on the action of these hormones or related substances during the prenatal period, 2) be suggestive evidence that the relationship between the neural tissues mediating mating behavior and the morphogenic fetal hormones parallels that between the genital tissues and the same hormones, and 3) direct attention to a possible origin of behavioral differences between the sexes which is ipso facto important for psychologic and psychiatric theory (18). Although comprehensive experiments have not yet been performed, initial investigations with an androgen have yielded effects which are so much more in line with current thought in the area of gonadal hormones and sexual differentiation (19, 20, 21, 22) than the earlier experiments on behavior, that the results are summarized here.

### Materials and Methods (the Production of Hermaphrodites)

Most of the experimental animals were born to mothers which had received intramuscular injections of testosterone propionate<sup>2,3</sup> during much of gestation. One group was composed of females in which there were no visible abnormalities of the external genitalia. These are referred to as the *unmodified females*. Their mothers were given an initial injection of 1 mg. of testosterone propionate some time between day 10 and day 27 after conception and 1 mg. every third or fourth day thereafter until the end of pregnancy.

The larger group was composed of females in which the external genitalia at the time of birth were indistinguishable macroscopically from those of their male siblings and untreated males. These animals are designated *hermaphrodites*. Laparotomy was necessary in order to distinguish these genetical females from males; it was performed within the first week after birth. Their mothers received an initial injection of 5 mg. of testosterone propionate on day 10, 15, 18, or 24 of the gestation period and 1 mg. daily thereafter until day 68.

Control animals were females and males from untreated mothers from the same stock as the experimental animals.

All these animals, i.e. the unmodified females, the hermaphrodites, their male siblings, and the control females and males, were used in four experiments designed to test the effects of testosterone propionate received prenatally on the responsiveness of the animals as adults to male and female hormones.

### Experimental

**Experiment 1** *The behavior of gonadectomized adult unmodified females and hermaphrodites injected with estradiol benzoate and progesterone.*

### Subjects

Fourteen females from untreated mothers.  
Fourteen unmodified females.

**Table 22.1**

Duration of heat and lordosis in gonadectomized guinea pigs given different amounts of estradiol and 0.2 mg. of progesterone

Subjects	Tests* N	Per Cent of Tests Positive for Estrus	Mean Latency in Hours	Mean Duration of Heat in Hours	Median Duration of Max. Lord. in Seconds
<i>1.66 µg.</i>					
Control females	19	89	5.7	5.7	11.5
Unmodified females	20	65	6.5	2.8	8.5
Hermaphrodites	9	22	8.5	2.5	2.0
Castrated males	8	38	6.0	1.2	2.0
<i>3.32 µg.</i>					
Control females	33	94	4.4	7.3	12.3
Unmodified females	38	68	5.6	2.8	5.1
Hermaphrodites	18	22	8.0	2.0	3.0
Castrated males	16	31	4.5	3.2	2.7
<i>6.64 µg.</i>					
Control females	28	96	3.7	7.2	9.3
Unmodified females	22	77	5.8	3.3	6.0
Hermaphrodites	18	22	9.2	2.0	2.0
Castrated males	16	0	—	—	—

\* All the animals were given one or more tests at each level of hormone.

Nine hermaphrodites.

Eight males from untreated mothers.

Except for four unmodified females gonadectomized when they were 45 days old, all the unmodified females and hermaphrodites were gonadectomized at 80 to 150 days of age. No data from the laboratory indicate that the response to exogenously administered sex hormones is influenced by age at the time of gonadectomy. The eight males were castrated before they were 21 days old.

### Tests

After gonadectomy, when the animals were 90 to 160 days old, tests were made of the responsiveness to 1.66, 3.32, and 6.64 µg. of subcutaneously injected estradiol benzoate followed 36 hours later by 0.2 mg. of progesterone.<sup>4</sup> Observations were continuous for 12 hours, beginning immediately after the injection of progesterone. Following the procedure of Goy and Young (4) hourly checks were made for the occurrence of the lordosis reflex in response to fingering. Individual records were kept of this measure of behavior and of the frequency of male-like mounting.

In three tests the control females, hermaphrodites, and males were observed for the occurrence of mounting in the absence of exogenous hormone. The unmodified females were given one such test.

The means and medians of the measures of behavior for which data were obtained were calculated from the individual averages and they are based on the data from the animals which responded to the hormones. For purposes of statistical analysis, maximum values (12 hours) for latency and 0 values for all other mea-

sures were arbitrarily assigned to the individuals failing to respond.

### Results

The data bearing on all the measures of the estrous response except mounting are summarized in table 22.1. The lower values for the per cent of tests positive for estrus, the mean duration of heat, and the median duration of the maximum lordosis were conspicuous effects of the treatment given prenatally and the differences among the groups are highly significant ( $P < .001$ ). Among the two groups of experimental females and the castrated males, the low guttural growl which is so characteristically a part of the pattern of lordosis in normal females, was commonly, and in some individuals always, lacking. Had the estimation of the duration of maximum lordosis been based only on complete responses, the differences among the groups would have been even greater.

Variations in medians for the duration of maximum lordosis were not systematically related to quantity of estradiol given prior to the tests. The analysis, therefore, was based on the medians of individual averages over all dosages. These medians were 11.3, 6.5, 2.3, and 2.5 seconds for control females, unmodified females, hermaphrodites, and castrated males, respectively. The median of the unmodified females, which most closely resembles that of the control females, is significantly different ( $U = 22$ ,  $P < .002$ ) from that of the controls.

Other differences also are indicative of the changes that were induced. Per cent response and duration of heat tended to increase in the control groups as the

**Table 22.2**

The quantity of mounting with and without estradiol and progesterone

Subjects	Mean Number of Mounts	
	Without Hormone	With Hormone*
Control females	0	10.7
Unmodified females	0	8.8
Hermaphrodites	4.4	5.6
Castrated males	11.8	16.7

\*Variation in the amount of mounting was not related to the quantity of estradiol. The means therefore are based on the averages for each individual whether the dosage was 1.66, 3.32, or 6.64  $\mu$ g. of the hormone.

quantity of injected estradiol was increased. Latency which is related inversely to the duration of heat (4) decreased. Among the experimental groups (unmodified females, hermaphrodites, and castrated males), similar relationships were seen only in the unmodified females.

In general the suppression of the capacity to display lordosis was proportional to the quantity of androgen injected prenatally. Amounts insufficient to alter external genital structures resulted in disturbances in the lordosis in only 50% of the animals, but the larger amounts that produced the hermaphrodites affected the lordosis in all. Within each group the effect on lordosis was not related to the quantity of androgen received prenatally. Among unmodified females, even siblings differed, one showing complete suppression of the lordosis and the other responding normally. The findings demonstrate that suppression of the capacity for displaying lordosis does not depend on masculinization of the external genitalia; clearly less androgen was required for the former than for the latter.

Additional evidence for the masculinizing effect of the prenatally administered androgen is provided by the data on the male-like mounting displayed by each group (table 22.2). When estradiol and progesterone were injected all groups displayed mounting, and the differences among the groups are not statistically significant. In contrast, on tests when no hormones were given, the hermaphrodites and castrated males were the only animals that mounted.

The interval from the beginning of the test to the display of mounting differed among the groups. Of the males which mounted, all did so at least once during the first hour. Of the 7 hermaphrodites which mounted, 5 or 71% mounted at least once during the first hour, but only 1 normal female (7%) and 1 unmodified female (7%) mounted this early in the test. The modal time for the onset of mounting was the 1st hour for the castrated males and hermaphrodites and the 6th and 7th hours for the control females and unmodified females, respectively. In this respect the hermaphro-

dites closely resembled the castrated males and seem to have been masculinized. The latency of mounting in the unmodified females was not different from that in the control females.

In one way the mounting performance of the unmodified females did differ from that of the controls. More unmodified than control females displayed mounting on tests after injections when the lordosis reflex could not be obtained. Of 8 unmodified females which failed to show lordosis, 6 or 75% mounted. Because of the small number of control females which failed to display lordosis after injection, older data on normal females from the same genetical stock are used for comparison. These data combined with those from the present study reveal that of 38 normal females failing to display lordosis after injection with comparable amounts of estradiol and progesterone only 4 or 10.5% mounted. The difference between the proportions of control females and unmodified females displaying mounting in the absence of lordosis is significant (C.R. = 4.02,  $P < .001$ ). Inasmuch as mounting was displayed spontaneously by the hermaphrodites, it was not possible with the animals available to determine the extent to which this behavior was being shown in response to the estradiol and progesterone.

## Conclusions

1. Prenatally administered testosterone propionate suppressed the capacity for displaying lordosis following gonadectomy and the injection of estradiol and progesterone. The effect was manifested either by an absence of lordosis or by a marked abnormality in its character when it was displayed.
2. Suppression of the capacity for displaying lordosis was achieved with a smaller quantity of the androgen than was necessary for the gross modification of the external genitalia.
3. The capacity to display male-like mounting was not suppressed.
4. Quantities of testosterone propionate sufficient to suppress lordosis and masculinize the genitalia also reduced the interval before mounting behavior was displayed.

**Experiment II** *Permanence of the effects of prenatally administered androgen.*

## Subjects

*Group 1* Three hermaphrodites used in the previous experiment.

*Group 2* Seven unmodified females used in the previous experiment.

*Group 3* Eight control females used in the previous experiment.

**Table 22.3**  
Behavioral responses to 3.32  $\mu$ g. of estradiol and 0.2 mg. of progesterone

		Tests at 6–9 Months of Age	Tests at 11–12 Months of Age
Hermaphrodites (Group 1)	Per cent response	33.0	0
	Latency to heat in hours	7.5	—
	Duration of heat in hours	2.5	—
	Median maximum lordosis in seconds	2.0	—
	Mean number of mounts	3.0	45.2
Unmodified females (Group 2)	Per cent response	55.0	71.0
	Latency to heat in hours	6.3	7.5
	Duration of heat in hours	2.2	2.3
	Median maximum lordosis in seconds	4.0	5.8
	Mean number of mounts	8.7	17.5
Normal females (Group 3)	Per cent response	95.0	94.0
	Latency to heat in hours	4.4	6.1
	Duration of heat in hours	7.2	4.5
	Median maximum lordosis in seconds	10.0	10.2
	Mean number of mounts	9.9	9.6

*Group 4* Six hermaphrodites injected with 500  $\mu$ g. of testosterone propionate per 100 gm. body weight per day from birth to 80 days of age.

*Group 5* Six normal females injected with the same amount of testosterone propionate from birth to 80 days of age.

*Group 6* Five mothers of hermaphrodites injected with testosterone propionate during pregnancy as described in Materials and Methods.

*Group 7* Eight untreated females comparable in age with those injected with testosterone propionate during pregnancy.

The animals in Groups 1 through 5 were gonadectomized when they were 80 to 150 days of age, those in Groups 6 and 7 when they were 1.5 to 3 years old. The operations on the animals in Group 6 were performed approximately 10 months after the last injection of testosterone propionate.

### Tests

All the animals received 3.32  $\mu$ g. of estradiol benzoate followed 36 hours later with 0.2 mg. of progesterone. The tests were similar to those given the hermaphrodites, unmodified females, and controls in Experiment I. The number, however, differed for each group and is shown in the description of the results. The values reported in the tables and the statistical treatment of the data were determined by the methods described in Experiment I.

### Results

The behavior of the 3 hermaphrodites, the 7 unmodified females, and the 8 control females is summarized in table 22.3 and compared with that displayed during

the earlier tests when the animals were 6 months old. The results reported in table 22.3 are based on at least 2 tests of each individual at each age level. No significant change occurred in the hermaphrodites and unmodified females for per cent response, latency to heat, duration of heat, and the duration of maximum lordosis. The normal females, however, showed a significant decrease in the duration of heat ( $T = 0$ ,  $P = .01$ ), reflecting perhaps a decrease in responsiveness to the hormones as the animals aged. The increase in mounting is significant for the unmodified females ( $T = 0$ ,  $P = .02$ ). The 3 hermaphrodites displayed increased mounting behavior, but the increase could not be evaluated statistically. Of the normal females, 3 showed increases, 3 a decrease, and 2 remained the same.

The contrast between the effects of prenatal and postnatal treatment is revealed by the results obtained from the animals treated neonatally (Groups 4 and 5) and from those treated during pregnancy (Group 6). During the period after withdrawal of the testosterone propionate, 5 of the 6 normal females which had been injected for 80 days after birth regained the ability to display lordosis, whereas the hermaphrodites did not (table 22.4). The effects of the postnatally administered androgen on the mounting behavior displayed by the animals in the two groups were complex and their presentation is being postponed until a further discussion can be given. The females treated with testosterone propionate while pregnant (Group 6) did not, like their "daughters," lose the capacity to display lordosis. Comparison of their behavior in response to estradiol and progesterone in five tests with that of untreated females of the same age (Group 7) (table 22.5), revealed that the differences between the groups are

**Table 22.4**

Per cent response, duration of heat, and maximum lordosis after cessation of treatment with testosterone propionate from birth to 80 days of age

		Approximate Age in Days at Time of Test			
		90	140	160	175
Hermaphrodites (Group 4)	Per cent response	0	0	0	0
	Mean duration of heat in hours	—	—	—	—
	Median maximum lordosis in seconds	—	—	—	—
Females (Group 5)	Per cent response	0	84	66	66
	Mean duration of heat in hours	0	4.6	1.7	3.7
	Median maximum lordosis in seconds	0	9.0	5.5	9.5

**Table 22.5**

Behavior of normal females treated with testosterone propionate for 50 days during pregnancy and tested 10 months later

	Per Cent Response	Latency of Heat in Hours	Duration of Heat in Hours	Mean No. of Mounts
Treated females (Group 6)	84	6.7	4.2	17.8
Untreated females (Group 7)	62	7.6	3.2	8.1

not significant for latency, duration of heat, and mounting.

### Conclusions

1. The suppression of the capacity for displaying the feminine components of the sexual behavior pattern which followed the administration of testosterone propionate prenatally appears to have been permanent.
2. Amounts of testosterone propionate which were effective prenatally had no conspicuous lasting effects when administered postnatally.

**Experiment III** *The behavior of gonadectomized hermaphrodites in response to testosterone propionate.*

### Subjects

Five hermaphrodites gonadectomized between 86 and 112 days of age.

Five normal females gonadectomized between 80 and 106 days of age.

Eight normal males castrated before 21 days of age.

When the animals were approximately 180 days old all received 2.5 mg. of testosterone propionate daily for 16 consecutive days.

### Tests

A sexual behavior test was given the day before the first injection. Additional tests were given on days 1 and 2 of the injection period, and every other day thereafter

until each animal had received 9 tests. The ninth test was given the day of the sixteenth injection.

### Results

The median value for mounting by the hermaphrodites and females in the single test prior to the injection of testosterone propionate was 0. For the males the median was 5.5.

The remaining data are summarized in table 22.6. They demonstrate the masculinizing effect of prenatally administered testosterone propionate on the female. Castrated males and hermaphrodites obtained the highest sexual behavior scores, the control females the lowest. The overall difference in scores was significant ( $P \sim .02$ ). The differences between the castrated males and hermaphrodites were not significant, whereas both groups differed significantly from the control females ( $P = .05$ ). The overall difference in the number of tests to the first display of mounting was significant ( $P < .01$ ). As with the sexual behavior scores, the difference between males and hermaphrodites was not significant, but both groups differed significantly from the control females ( $P = .02$ ). There was a significant overall difference ( $P < .01$ ) in the amount of testosterone propionate required before the first appearance of mounting. Again, the hermaphrodites resembled the castrated males in that there was no significant difference between these two groups, but both groups displayed mounting with significantly less hormone ( $P = .02$ ) than the control females.

### Conclusions

1. Adult hermaphrodites gonadectomized and injected with testosterone propionate were more responsive to this hormone than gonadectomized normal females.
2. The earlier appearance and greater strength of masculine behavior by the hermaphrodites given testosterone propionate are believed to be effects of the prenatally administered testosterone propionate on the tissues mediating masculine behavior and therefore to be expressions of its organizing action.

**Table 22.6**

Masculine behavior in gonadectomized adult animals injected with testosterone propionate

Group	Mean Sexual Behavior Score	Mean Mounts Per Test	Median Number of Tests to the First Display of Mounting	Median mg. of t.p. Prior to the Display of Mounting
Spayed untreated females	2.1	5.8	7.0	30.0
Spayed hermaphrodites	3.6	15.4	3.0	10.0
Males castrated prepuberally	5.0	20.5	1.5	3.8

**Experiment IV** *The behavior of adult male siblings of the hermaphrodites.*

### Subjects

Five males from untreated mothers.

Five males born to mothers receiving testosterone propionate during pregnancy. No hormone was administered after birth.

Five males born to mothers receiving testosterone propionate during pregnancy. These animals received 500  $\mu$ g. of the hormone per 100 gm. body weight daily beginning 1 to 3 days after birth and continuing 80 to 90 days.

### Tests

Five tests were given when the animals were 11 months old. In a test the subject was placed with a receptive female of approximately the same size, and the frequency of the display of selected measures of behavior was recorded for a maximum of 10 minutes. These measures included sniffing and nibbling, nuzzling, abortive mounting, mounting, intromissions, and ejaculation. A description of the measures and the method for computing scores are given by Valenstein, Riss and Young (23).

### Results

The mean scores are summarized in table 22.7. It is clear that any effect of the exogenous testosterone propionate was slight. There was no evidence of suppression of the capacity to display masculine behavior, if anything, the animals receiving the hormone prenatally achieved higher scores than the controls.

### Conclusion

The sexual behavior of adult males which had received testosterone propionate prenatally was not significantly different from that of untreated controls.

### Discussion

The data from the four experiments summarized in the preceding sections support the hypothesis that andro-

**Table 22.7**

Mean sexual behavior scores obtained by the three groups of adult males

Groups*	Tests				
	I	II	III	IV	V
Untreated	6.9	6.6	9.2	7.2	10.4
Testosterone propionate prenatally	10.4	9.3	9.1	9.3	12.2
Testosterone propionate prenatally and postnatally	10.9	11.2	7.3	11.1	9.4

\* Difference among the groups not significant;  $F = 1.30$ ;  $df = 2, 12$ .

genic substances received prenatally have an organizing action on the tissues mediating mating behavior in the sense of altering permanently the responses females normally give as adults. This possibility was suggested by the work of Dantchakoff (13, 14, 15), Raynaud (16), and Wilson, Young and Hamilton (17). Probably, however, because interest in the role of gonadal hormones in the regulation of mating behavior was concentrated so largely on the neonatal individual and adult, the suggestion was never incorporated in our theories of hormonal action. This step may now be taken, but when what has been learned from the present investigation is related to what has long been known with respect to the action of androgens on the genital tracts, a concept much broader than that suggested by the older studies is revealed.

The embryonic and fetal periods, when the genital tracts are exposed to the influence of as yet unidentified morphogenic substances (19, 20, 21, 22, 24), are periods of differentiation. The adult period, when the genital tracts are target organs of the gonadal hormones, is a period of functional response as measured by cyclic growth, secretion, and motility. The response depends on whether Müllerian or Wolffian duct derivatives have developed, and although generally specific for hormones of the corresponding sex, it is not completely specific (25). For the neural tissues mediating mating behavior, corresponding relationships seem to exist. The embryonic and fetal periods are periods of organization or "differentiation" in the direction of masculinization or feminization. Adulthood, when gonadal

hormones are being secreted, is a period of activation; neural tissues are the target organs and mating behavior is brought to expression. Like the genital tracts, the neural tissues mediating mating behavior respond to androgens or to estrogens depending on the sex of the individual, but again the specificity is not complete (26, 27).

An extension of this analogy is suggested by the work done on the embryonic differentiation of the genital tracts, particularly that by Burns and Jost and summarized in their reviews (20, 21, 22). It will be recalled from the data reported in the present study that testosterone propionate administered prenatally affected the behavior of the male but slightly, whereas the effects on the female were profound. Not only was there a heightened responsiveness to the male hormone as revealed by the stronger masculine behavior displayed when testosterone propionate was given, but there was a suppression of the capacity to display the feminine components in response to treatment with an estrogen and progesterone. In studies of the genital tracts there were no effects on the male except for a slight acceleration in the development of the prostate and seminal vesicle and an increase in the size of the penis (28). Within the female, the Wolffian duct system was stimulated (13, 14, 15, 28, 29), and locally, when a fetal testis was implanted into a female fetus (20, 21), there was an interruption of the Müllerian duct on that side. What has not been seen when an exogenous androgen was administered, except by Greene and Ivy (30) in some of their rats, is a suppression or inhibition of the Müllerian duct system corresponding to the suppression of the capacity for displaying the feminine component of behavior.

The failure to detect a corresponding suppressing action on the Müllerian duct does not exclude the possibilities 1) that such an effect will be found, and 2) that the suppressing action is in the nature of a reduction in the responsiveness of the genital tract to estrogens rather than in the inhibition of its development. Such an effect was encountered in rats given testosterone propionate prenatally (17) when it was found that uterine as well as behavioral responses to estrogen and progesterone were suppressed.

A final suggestion with respect to the analogy we have postulated arises from a comparison of our results with those reported by Dantchakoff and Raynaud. These investigators stressed the increased responsiveness of their masculinized guinea pigs and mice to exogenous androgens, and seemed to regard the change as the expression of an inherent bisexuality. The possibility that there might have been a suppression of the capacity to respond as females and therefore an inequality of potential does not seem to have been con-

sidered. Like Dantchakoff (13, 14, 15), Raynaud (16), and many others (9, 31, 32, 33, 34, 35), the existence of a bisexuality is assumed. We suggest, however, that in the adult this bisexuality is unequal in the neural tissues as it is in the case of the genital tissues. The capacity exists for giving behavioral responses of the opposite sex, but it is variable and, in most mammals that have been studied and in many lower vertebrates as well, it is elicited only with difficulty (27). Structurally, the situation is similar. Vestiges of the genital tracts of the opposite sex persist and are responsive to gonadal hormones (36, 37), but except perhaps in rare instances, equivalence of organs and responses in a single individual is not seen (36, 37, 38, 39).

The concept of a correspondence between the action of gonadal hormones on genital tissues and neural tissues contains much that is new and its full scope is not yet clear. The possibility must be considered that the masculinity or femininity of an animal's behavior beyond that which is purely sexual has developed in response to certain hormonal substances within the embryo and fetus.

Thus far the permanence of the effect achieved when testosterone propionate was received prenatally has not been achieved when the same hormones were administered to adults or to newborn individuals. The dependence of this "permanence" on the action of the hormone during a possible critical period must be ascertained.

The nature of the modifications produced by prenatally administered testosterone propionate on the tissues mediating mating behavior and on the genital tract is challenging. Embryologists interested in the latter have looked for a structural retardation of the Müllerian duct derivatives culminating in their absence, except perhaps for vestigial structures found in any normal male. Neurologists or psychologists interested in the effects of the androgen on neural tissues would hardly think of alterations so drastic. Instead, a more subtle change reflected in function rather than in visible structure would be presumed.

Involved in this suggestion is the view that behavior may be treated as a dependent variable and therefore that we may speak of shaping the behavior by hormone administration just as the psychologist speaks of shaping behavior by manipulating the external environment. An assumption seldom made explicit is that modification of behavior follows an alteration in the structure or function of the neural correlates of the behavior. We are assuming that testosterone or some metabolite acts on those central nervous tissues in which patterns of sexual behavior are organized. We are not prepared to suggest whether the site of action is general or localized.

## Notes

1. This investigation was supported by research grant M-504 (C6) from the National Institute of Mental Health, Public Health Service.
2. Testosterone propionate (Perandren propionate) was supplied by Ciba Pharmaceutical Products, Inc.
3. The injections were made by Mr. Myron D. Tedford, a Public Health Service Predoctoral Fellow, who is using these and other animals treated similarly for a study of the structural changes in the gonads, genital tracts, and external genitalia, and the course of gestation. We are indebted to him for supplying us with the animals whose behavior was investigated.
4. Estradiol benzoate (Progynon-B) and progesterone (Proluton) were supplied by the Schering Corporation.

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Research on the relationships between the hormones and sexual behavior has not been pursued with the vigor justified by the biological, medical, and sociological importance of the subject. Explanation may lie in the stigma any activity associated with sexual behavior has long borne. In our experience, restraint has been requested in the use of the word *sex* in institutional records and in the title of research proposals. We vividly recollect that the propriety of presenting certain data at scientific meetings and seminars was questioned. Counteracting this deterrent is the stimulation which has come from colleagues in many disciplines to whom we have appealed for help, and the satisfaction we have felt in seeing a picture emerge as the pieces of the puzzle have been studied and fitted together.

#### Relationships in the Adult

Causal connections between gonadal hormones and the development of the capacity of infrahuman vertebrates to display sexual behavior have long been assumed, although the existence of such relationships in man is questioned (1–3). Doubt has also been expressed that a specific relationship exists between any one hormone (or class of hormones) and the behavior it facilitates in adults in general, from fish to man (4–6).

A number of explanations may be given for the uncertainty which exists. Human sexual activity is influenced by many psychologic factors, the social level, cultural background, and tradition. The many reports are not completely trustworthy. Physiological correlates with individual behavior are largely nonexistent, and controlled study in man as we know it in laboratory animals is impossible. In our opinion the many differences in behavior which in the growing child and adult are socially rather than hormonally determined have obscured the possible role of the hormones in maintaining the strength of the sexual drive. Even in lower mammals the same quantity of hormone elicits almost as many modes of response as there are individuals. This fact may have contributed to the doubt, to which we have alluded, that there is any great degree of hormonal specificity. In the human female, sexual

responsiveness does not have the sharp relationship to folliculogenesis and to the functioning of the corpus luteum in the ovary that it does in most lower mammals (7). The degree to which this evolutionary change within the primates has been accompanied by an emancipation from the effects of hormonal action is not known.

The need for testicular androgen in the maintenance of sexual vigor in the male has been questioned by some students of the problem. In man (5, 8), the dog (9), the domestic cat (10), fishes (6), and birds (11), there is, in males, a persistence of sexual activity for some weeks or months after castration which has not been explained satisfactorily; a corresponding persistence is encountered rarely if at all in females below the primates. The restoration of sexual vigor by replacement therapy also requires weeks in the male and only hours or days in the female. The longer time lapse which occurs, regardless of the direction of hormonal change, suggests that the manner of hormonal action in the male is greatly different from that in the female rather than that the strength of sexual behavior is independent of the presence of testicular androgen.

Finally, in this brief consideration of the subject, there are the important studies of deviant sexual types by Hampson and Hampson (1), Money (2), and the recent report by Völkel (3). The data these clinical investigators collected led them to conclude that the establishment of gender role or psychologic sex can be independent of chromosomal sex, gonadal sex, hormonal sex, internal reproductive structures, and external genital morphology. They relate the process rather to “the many experiences of growing up, including those experiences dictated by his or her own bodily equipment” (1).

The interest of one of us (W.C.Y.) in the relationship of the hormones to sexual behavior goes back to an observation made during his graduate years at the University of Chicago when he was looking for signs that would be useful in the identification of female guinea pigs in heat. No active interest was taken, however, until more than 6 years later. During a lull at Brown University, Young, Hugh I. Myers, and Edward W.

Dempsey, while waiting for what turned out to be the disapproval of an application for a small amount of money for work on the function of the epididymis, fell into a discussion of the abrupt and dramatic change that occurs in the behavior of the female guinea pig when she comes into heat. They wondered whether this change is associated with any structural change in the ovaries. Continuous day and night observation of the laboratory animals for several months was rewarded by the information Young and his co-workers were seeking. The beginning of heat was found to coincide closely with the beginning of the preovulatory growth phase of the Graafian follicle (12); it could be that the three investigators, none of whom had any training in psychology, had stumbled on the only spontaneously occurring macroscopic structural change associated with the alteration of a behavioral state in a mammal.

The reports that ovariectomized mice, rats, rabbits, and dogs copulated after the injection of follicular fluid or of the estrogens that were available at that time suggested that the same behavior would occur in the guinea pig. To the surprise of Young and his associates, irregular results were obtained. These led to the conclusion that a second substance must participate. With the help of Roy Hertz, who worked with the group that year, Dempsey took his cue as to the nature of this substance from the demonstration that the preovulatory growth phase in anestrus cats is stimulated by hypophyseal luteinizing hormone (13). Tests soon revealed that this gonadotrophin does not produce heat directly; they suggested that by stimulating preovulatory swelling, ovulation, and production of progesterone luteinizing hormone leads indirectly to the display of estrous behavior by animals previously injected with an estrogen (14). The progesterone as it turned out, was the second participating substance. Its synergistic action in combination with estrogens to bring latent mating behavior to expression has since been demonstrated in an impressive number of mammalian species (see 7). Astwood (15) showed later that the hypophyseal gonadotrophin responsible for the production of progesterone is luteotrophin rather than luteinizing hormone.

### Importance of Soma

The familiarity obtained with the behavior of the female guinea pig and later with that of the rat and male guinea pig revealed (i) that in repeated tests individual differences in behavior were remarkably consistent and reliable (16, 17); (ii) that in the female these differences, except perhaps for the male-like mounting behavior, are not related to the number of rupturing Graafian follicles (18, 19); and (iii) that in neither sex are the dif-

ferences in the vigor of the behavior related to the quantity of administered hormones, provided of course a threshold has been exceeded (17, 20). These findings led to the realization that the nature of the latent behavior brought to expression by gonadal hormones depends largely on the character of the soma or substrate on which the hormones act (19). The substrate was assumed to be neural (9). Unknown to Young and his co-workers until 15 or 20 years later, Goodale (21) in 1918 had been impressed by the failure of ovaries implanted into capons to feminize their behavior and had written, "the character of sexual reactions seems to depend upon the substratum, while the gonad merely determines that it shall be given expression."

### Factors Influencing Character of Soma

It follows from this principle that an investigator trying to account for the behavioral differences between individuals, instead of looking to the gonadal hormones, would do better to look to the factors which influence the character of the tissues on which these hormones act. The age of the animal was one of the first factors investigated, and data indicating that age is significant were obtained immediately. Responsiveness or reactivity of the tissues to injected gonadal hormones is lacking during early infancy and increases gradually to the level observed in the adult (22, 23).

The possibility of further changes as aging progresses has not been investigated. The thyroid was thought of as a factor influencing responsiveness, and Young and his co-workers found that female guinea pigs surgically thyroidectomized and given  $I^{131}$ , to suppress any accessory thyroid activity, ovariectomized, and injected with estradiol and progesterone were less responsive to the latter substances than control females (24). The many reports of the effects of thyroid hormone on the vigor of sexual behavior in the male are so contradictory (7) that prediction of the relation of this hormone to the animal's responsiveness to androgens, before adequately designed experiments have been carried out, would be unwise.

The belief that the genetic background is an influential determinant of the character of the soma was soon confirmed. Intact male and female guinea pigs of the highly inbred strains 2 and 13 exhibit significant differences in their behavior. The differences are displayed consistently after gonadectomy and injection of the same amounts of the appropriate hormones (20, 23, 25). The hereditary basis of sexual behavior was studied. For both male and female behavior a high degree of heritability was demonstrated. The inheritance is autosomal, of the sex-limited or sex-influenced type, and appears to be polygenic for most of the behavioral characteristics studied. Sexual behavior is not inherited

as a unitary trait, and the elements composing the patterns of behavior show a surprising degree of independence of one another. In the male, phenotypic dominance of strain 13 was found for specific behavioral characteristics—for example, frequency of mounting. With respect to other characteristics, such as latency to ejaculation, strain 2 was dominant (26). In the female, the characteristics of frequent male-like mounting, vigorous lordosis, and responsiveness to injected estrogen appeared to have independent modes of inheritance and separate genetic bases (27).

Attention was drawn to the possibility that experiential or psychologic factors might have a role in the determination of the character of the soma by two young psychologists in the laboratory, Elliott S. Valenstein and Walter Riss, who could not accept the view that inheritance was accounting for the entire action. Again, the hunch was a good one. In a relatively short time after they directed their attention to the behavior of males raised in isolation from the day of birth, except for association with the mother, the necessity of contact with other animals for the maturation of normal patterns of sexual behavior was demonstrated (28). These males were sexually aroused to the same degree as normal males and attempted to mount frequently. However, the males that had been raised in isolation displayed an inability to properly mount and clasp a female. Presumably as a result of this inability, intromission was rarely achieved. These behavioral deficiencies characteristic of males reared in isolation were not overcome by injections of testosterone propionate and therefore cannot be attributed to a hormonal deficiency. The effect of isolation on maturation of the behavior of the female is less pronounced, except, interestingly, that isolation has an inhibiting effect on the male-like mounting behavior displayed so commonly by the female guinea pig (29). The guinea pig is not alone in needing contact with other animals for the maturation of normal behavior. This need has been demonstrated many times in species as widely separated phylogenetically as ring doves, domestic turkeys, rats, cats, rhesus monkeys, chimpanzees, and man (10, 30).

### Special Influence of Early Hormonal Factors

Up to this point nothing in the work with young or adult animals had suggested that gonadal hormones serve to organize the tissues mediating sexual behavior in the sense of differentiation, as experimental embryologists use the word. Conceivably the action is organizational before birth or before sexual maturation, and activational in the adult.

We were aware that numerous investigators have obtained a full functional sex reversal (including breed-

ing) in fishes and amphibians after administering heterotypical hormones during the embryonic and larval stages [see 31 for a few of the many reports reviewed by Young (32)]. More important for our thought was the statement by Dantchakoff (33) that female guinea pigs given testosterone prenatally had ovaries and two sets of duct systems. Oviducts, uterus, and vagina existed along with epididymides, ducti deferentes, seminal vesicles, prostate, Cowper's glands, and a penis, all differentiated and developed to varying degrees. An inverse relationship was found between penile structure and the degree of vaginal development. After injections of testosterone, masculine behavior was displayed. A repetition of Dantchakoff's experiment was dictated by the circumstance that no controls seem to have been used in her studies of this species, in which most normal females display male-like mounting as a part of the estrous reactions. Once a satisfactory method of administering the hormone had been developed by Myron D. Tedford, whose interest was mainly in the structural changes, pseudohermaphroditic females were produced routinely. The genital tracts were similar to those described by Dantchakoff, although probably encompassing a larger range of variations in structure. A marked display of masculine behavior was seen, as well as a lowered capacity to display feminine behavior (34)—an effect not observed by the earlier workers. Loss of the ability to come into heat was greatest when androgen treatment was started on day 30 of the 67- to 71-day gestation period, regardless of the duration of treatment and the total amount of androgen (35) (table 23.1).

For us, these results produced an exciting moment. It was clear, first, that the gonadal hormones, or at least testicular androgens, have a dual role in the con-

**Table 23.1**

Loss of the ability of female guinea pigs to come into heat relative to the amount of androgen (testosterone propionate) injected in the prenatal period and to the length of the period of treatment

Period of Prenatal Injection of Androgen (Days)	Total Amount of Androgen Injected (mg)	Percentage Failing to Come into Heat
15 to 30	40	0
15 to 40	50	12
15 to 45	55	36
15 to 60+	70+	27
20 to 65	70	45
25 to 40	40	23
30 to 45	40	67
30 to 55 or 65	50 or 60	92 or 91
35 to 65	55	60
40 to 65	50	33
50 to 65	40	0

trol of sexual behavior in the guinea pig. During the fetal period the hormones have an organizing action on the neural tissues destined to mediate mating behavior after the attainment of adulthood; during adulthood their role is one of activation. In other words, during fetal morphogenesis androgens exert a fundamental influence on the organization of the soma, determining whether the sexual reactions brought to expression in the adult will be masculine or feminine in character.

Second, it was clear that the rules of hormonal action are identical with those shown by the experimental embryologists to be applicable to the genital tracts (36). During the fetal period the gonadal hormones influence the direction of differentiation. During adulthood they stimulate functioning, be it contraction of smooth muscle fiber, secretion of epithelial cells, or endometrial sensitization for implantation.

The comparison can be extended. Evidence has been presented by experimental embryologists that, as the male develops, fetal testicular hormone is responsible for differentiation of the Wolffian duct system (precursor of the male genital tract) and suppression of the Müllerian duct system (precursor of the female genital tract). In the female and in the castrated fetal male, in both of which testicular androgen is absent, there is development of the Müllerian duct system and regression of the Wolffian duct system. In our experiments the administration of an androgen to developing female fetuses was followed by the production of individuals in which there had been a stimulating action on the tissues (presumably neural) having the potential capacity for mediating masculine behavior, and a suppressing action on the tissues destined ordinarily to mediate feminine behavior.

In order to complete the analogy it was necessary to demonstrate that genotypic males castrated before the end of the period in which the organizing action of the fetal testicular hormone ordinarily occurs would display feminine behavior as adults. For us, such an operation on the young fetal guinea pig was not feasible. The best available evidence from tests of fertility and mating behavior indicated that this organizational period in the rat, a species with a short period of gestation, is postnatal (37) rather than prenatal, and that it ends at approximately the 10th day after birth. If the analogy could be extended, male rats castrated during this short period after birth should display feminine behavior, or at least elements of feminine behavior, when injected with estrogen and progesterone as adults.

An experiment designed to test this hypothesis has just been completed by Kenneth L. Grady, a graduate student. Male rats were castrated at 1, 5, 10, 20, 30, 50, and 90 days of age, and as a criterion, females were ovariectomized at 90 days. When they were 120 days

**Table 23.2**

Mean copulatory quotients (lordosis/mounts) for rats gonadectomized at different ages and tested after injection of estradiol and progesterone at 120 days

Day of Gonadectomy	Quotient
<i>Females</i>	
90	0.619
<i>Males</i>	
1	0.436
5	.218
10	.014
20	.029
30	.042
50	.026
90	.019

old, and from then on, all animals were tested, after injections of estradiol and progesterone, for the display of feminine behavior in response to mounting by intact males. As we had expected, the experimental males displayed feminine behavior. Those castrated on day 1 or day 5 displayed significantly more receptive behavior than those castrated as late as day 10. Castration later than day 10 did not promote the retention or development of female behavioral characteristics (38) (see table 23.2).

Tests of masculine behavior in male rats castrated soon after birth and tested as adults are currently in progress. When these tests are completed the model established by the experimental embryologists will have been duplicated for behavior. The results may be anticipated from an early study in which male rats castrated on day 1 exhibited a marked deficiency in copulatory ability as compared with those castrated on day 21 or later (39). If this proves to be a representative finding, our work on the two sexes will have produced complementary pictures of the organizational influences of androgen. On the one hand, females treated with androgen during the appropriate period show a regression or inhibition of feminine behavior and an accentuation of masculine behavioral traits. Males deprived of the principal source of endogenous androgen during a comparable period show accentuated feminine behavior and the absence of, or a greatly diminished capacity for, masculine behavior.

#### Extension to Sex-Related Behavior

When Phoenix, Goy, Gerall, and Young (34) were summarizing their data on the behavior of the female pseudohermaphroditic guinea pigs, they suggested that the organizing or sex-differentiating action of fetal gonadal substances may affect behavior beyond that which is primarily sexual in the sense of being directed

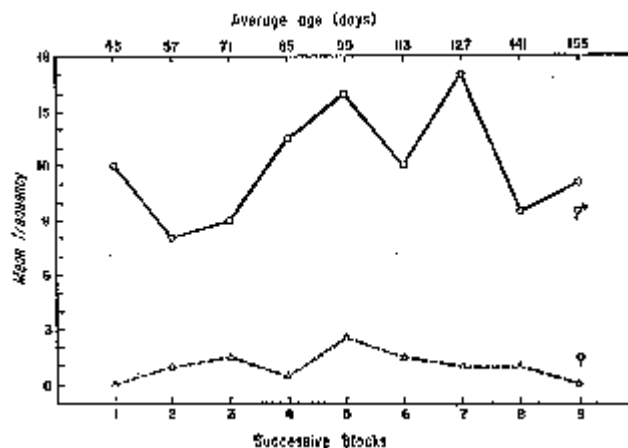
**Figure 23.1**

Female pseudohermaphrodite produced by injecting testosterone propionate into the mother during pregnancy. The treatment involved injection of 25 mg daily from post-coital day 40 through day 50; 20 mg from day 51 through day 70; and 10 mg from day 71 through day 90. There were no injections during the balance of the 166-day period of gestation. A prominent and well-formed phallus is visible to the right of the empty scrotal fold. The surgical scar in the right inguinal region resulted from a laparotomy which showed that there was no testis.

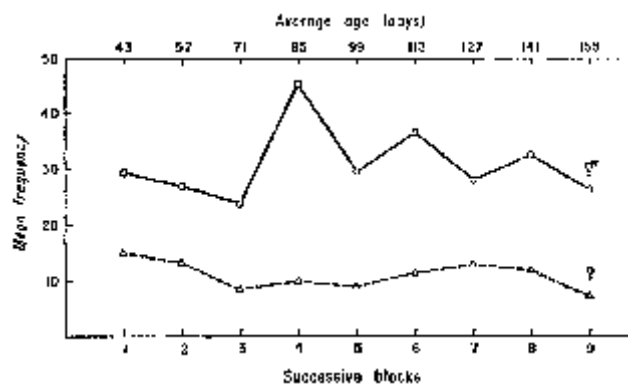
solely toward the attainment of sexual aims. The rhesus monkey seemed better suited than the guinea pig or rat for a test of this hypothesis, so we proceeded accordingly.

Although an androgen treatment entirely compatible with the maintenance of pregnancy has not been worked out, we have succeeded in producing three female pseudohermaphroditic subjects with conspicuous genital alterations (figure 23.1). Two have been studied in considerable detail. We based our study of the early social patterns displayed by these individuals on the model established by Leonard Rosenblum during his graduate training at the University of Wisconsin. Accordingly, the pseudohermaphroditic females were allowed unrestricted social interaction with two untreated females for 20 minutes per day, 5 days per week, in a specially designed play room.

The results from 90 such observational sessions, covering the second through the fifth months of life, have been analyzed recently. A number of social behaviors, known to be sexually dimorphic and without any immediate instrumentality relative to mating, appear to have been influenced in the masculine direction by our prenatal treatments with androgen. The social behavior of the untreated females did not differ importantly from that described for normal females by Rosenblum, but the behavior of the treated females much more closely resembled his description of that of males. The pseudohermaphroditic females threatened, initiated

**Figure 23.2**

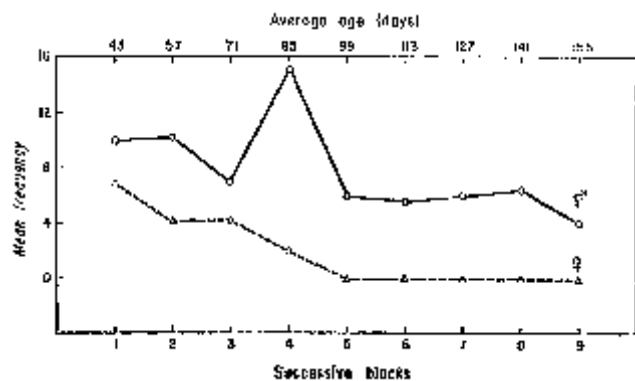
Display of facial threat by female pseudohermaphroditic (solid line) and normal female (broken line) monkeys plotted relative to age. The abscissa is scaled in successive blocks of five trials.

**Figure 23.3**

Invitation to play by female pseudohermaphroditic (solid line) and normal female (broken line) monkeys, plotted relative to age. The abscissa is scaled in successive blocks of five trials.

play, and engaged in rough-and-tumble play patterns more frequently than the controls (figures 23.2–23.4). Like the males studied by Rosenblum, these pseudohermaphrodites also withdrew less often from the initiations, threats, and approaches of other subjects.

Analysis of the sexual behavior displayed by these pseudohermaphroditic females, although far from complete, already shows that it is not only in their patterns of withdrawing, playing, and threatening that they display a bias toward masculinity. In special tests with pairs of females, one pseudohermaphroditic and one normal, the pseudohermaphrodites consistently displayed more frequent attempts to mount, regardless of whether the normal female was brought to the hermaphroditic female's cage or vice versa. Their attempts to mount, while infantile and poorly oriented, are beginning to be integrated with pelvic thrusting and even, on a few occasions, phallic erection.



**Figure 23.4**

Rough-and-tumble play by female pseudohermaphroditic (solid line) and normal female (broken line) monkeys, plotted relative to age. The abscissa is scaled in successive blocks of five trials.

Our work with the primates has not progressed to a point where it may be considered definitive, partly because of the limited duration of the study and partly because of the very small number of subjects. We nevertheless consider the results to be highly encouraging and supportive of the general conclusions developed in our more extensive studies with the infraprimates mammals, concerning the action of the gonadal hormones.

### Implications

Implied in the present discussion of data bearing on the organizing action of androgen on the neural tissues destined to mediate sexual behavior is the view that a part or parts of the central nervous system are masculine or feminine, depending on the sex of the individual. This concept is not new; it has been developing over the years in the writings of investigators whose approach has been entirely different from ours (4, 40). What we have found adds support to this view and suggests something of the way in which masculinity or femininity is conferred on the nervous system.

An additional thought merits attention. It is that the principles of hormonal action in effecting this sexual differentiation of the developing brain provide a model to which we may look for a reexamination of the psychosexual incongruities discussed by Hampson and Hampson (1) and by Money (2) in their reviews and in their many articles published since the middle 1950's. We accept and, in our own work with lower mammals, have documented the importance of the subject's experience. However, explanation of the cases these investigators present need not lead to a rejection of the concept of a predetermined psychosexuality for the concept of a psychologic sexual neutrality at birth. If the endocrinology of the differentiation of the capacity to display masculine and feminine sexual behavior as we have worked it out for the guinea pig and the rat is

applicable to man, the incongruities in the patients these workers examined can be explained without postulating a psychosexual neutrality at birth and attributing the gender role and sexual orientation solely to the individual's life experiences while growing up. In view of what we have learned an endocrinological basis which is consistent with the concept of psychologic bisexuality exists for the interpretation of most if not all of the cases they report. This is true of their hermaphrodites with ambiguous or masculinized external genitals and with a female sex chromatin pattern (comparable with our female pseudohermaphrodites); of the cryptorchid hermaphrodites with a male sex chromatin pattern, in which testicular function was clearly subnormal; of the simulant females with a male sex chromatin pattern but with the as yet unexplained feminizing testes; and of the "females" born with gonadal dysgenesis and an XO or XY chromosome pattern (Turner's syndrome). The large group of hyperadrenocortical patients with the female sex chromosome pattern probably would fit into this picture were the circumstances such that more information about the parameters of the excessive androgen production could have been obtained.

We realize as we venture into a clinical area that a question exists regarding the extent to which what we have found in the guinea pig, rat, and monkey is applicable to man. We call attention, however, to an interesting similarity revealed by Milton Diamond during his graduate work at the University of Kansas. After noting that testosterone does not induce masculinization of the adult female guinea pig while it is pregnant, he examined the clinical literature and found an apparent comparable lack of masculinization in pregnant human beings: 27 of 31 women who received androgenic hormones or gestagens in quantities sufficient to masculinize the female fetuses were not themselves virilized (41).

### Direction of Future Research

As we have proceeded with our analysis of the relationships of the hormones to sexual behavior and, more recently, to sex-related behavior, we have always been aware that many questions remain to be answered. At the same time a picture is emerging, and, with good fortune in the selection of materials and techniques, much more of it will be revealed in the future.

Without discounting the influence of psychologic factors, which we know is great, or the need for carefully recorded observations of behavior, we expect that, increasingly, the materials and techniques used will be those of the neurologist and the biochemist. The directions many neurologists are taking are indicated by the various reports of efforts to locate the neurological sites

of hormonal action and define the pathways of stimuli for the many responses that are given (see 42).

Few biochemists have been attracted to the problem, but it is they who must clarify the mechanisms of hormonal action in organizing the tissues of the central nervous system during development and in bringing behavior to expression in the adult. They may be helped in such a search by the circumstance that cellular elements in the genital tracts, which differentiate and are activated under the influence of these same hormones, are at present more accessible for histophysiological study than those in tissues of the central nervous system. It is to be hoped that clues will come from the work of the many investigators whose studies are described in recent reviews (43).

The need for studies of sexual behavior in man is great. Methods for collecting trustworthy, meaningful data and means of ascertaining whether the many behavioral states are associated with hormonal action in the developing fetus and in the adult should be worked out. The possibility that such relationships exist and that typical and deviant behaviors have a physiologic as well as a psychologic basis may no longer be excluded.

### Summary

From an attempt made 30 years ago to attain a limited objective, we have proceeded with what turned out to be a long-term investigation. Evidence has accumulated indicating that the gonadal hormones have a broad role in the determination of behavior. We have long known that they act to bring sexual behavior to expression, certainly in adult vertebrates below man. We now know, in addition, that during a period of organization and differentiation which is prenatal in the guinea pig and monkey and postnatal in the rat, the hormones act according to principles which appear to be identical with those operative during the differentiation of the genital tracts, and they effect a corresponding differentiation or organization of neural tissues.

The data thus far accumulated from a study of the behavior of two female pseudohermaphroditic monkeys suggest that this early hormonal action is also responsible for the establishment of much of the sex-related behavior which is a part of the masculinity or femininity of an individual but which is not related directly to the reproductive processes.

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44. During the years in which the investigations discussed were in progress at Brown University, the Yale Laboratories of Primate Biology, and the University of Kansas, support was provided by the National Research Council's Committee for Research in Problems of Sex, and by grants, particularly MH-00504, from the National Institute of Mental Health, Bethesda, Md. Dr. Leon H. Schmidt, who, at the time of this work was director of the Christ Hospital Institute for Medical Research, and Dr. Harry F. Harlow, director of the Wisconsin Primate Research Center, extended the use of facilities in their laboratories for the production and study of female pseudohermaphroditic monkeys. Testosterone propionate (Perandren) was generously supplied by CIBA Pharmaceutical Corporation, Summit, N.J.

The most predominant social behavior to be observed among prepubescent rats is that of social play or play-fighting. This behavior has been found to occur more frequently in male pups than in female pups (Meaney and Stewart, 1981; Olioff and Stewart, 1978; Poole and Fish, 1976). This form of social play, often referred to as rough-and-tumble play, has been reported to be male predominant in several other species including hamsters (Goldman and Swanson, 1975), rhesus monkeys (Goy and Goldfoot, 1974; Harlow, 1969), baboons (Owens, 1975), and humans (Blurton-Jones, 1976).

Working with rhesus monkeys, Goy and Goldfoot (1974) found that females exposed to exogenous testosterone propionate (TP) during gestation engaged in rough-and-tumble play at rates comparable to those of their male peers. Similarly, Olioff and Stewart (1978) found that the administration of TP to female rats on postnatal Days 1 and 2 was sufficient to eliminate the sex difference in social play.

One problem of concern to those studying the role of testicular hormones in the determination of behavior is to specify which of testosterone (T) or its major metabolites is primarily responsible for any observed behavior difference between the sexes. This is a question of particular interest in view of the major role attributed to the metabolite  $17\beta$ -estradiol ( $E_2$ ) in the differentiation of the neural circuitry involved in gonadotropin release and in the defeminization and masculinization of sexual behavior patterns in many rodents.  $E_2$  is known to be converted from T in many regions of the brain during the period of differentiation and to bind with high affinity to estrogen receptors within specific brain cells. It is this feature that has made it possible to begin to relate hormone action to changes in specific brain structures during this period. No such direct central nervous system action for T itself or for its  $5\alpha$ -reduced and nonaromatizable metabolite, dihydrotestosterone (DHT), has yet been determined, though it has been suspected. Thus while it is well recognized that DHT plays the major role in promoting the development of male genitalia and body type, the evidence

that exists for direct androgenic action in the central nervous system during differentiation is considered equivocal. One reason for this is that until recently receptors for T or DHT were not known to be present in the brain of developing animals during critical periods for differentiation. The finding of androgen receptors in the limbic brain of 1-day-old rats, receptors that exhibit nuclear binding properties typical of androgen receptors in adult rats, is therefore of great interest (Fox, Vito, and Wieland, 1978; Lieberberg, Maclusky, Roy and McEwen, 1978; Vito, Wieland, and Fox, 1979). Furthermore, the report of  $5\alpha$ -reductase activity in the brain of neonatal rats adds to the possibility that androgen metabolites of T may play a role in the differentiation of neural tissue (see Martini, 1978). In this chapter we report on the results of six experiments designed to investigate the influence of T and some of its metabolites on the social play of prepubescent rats.

The animals were studied during the prepubertal period from Days 26 to 40 of age, except in Experiment 2 in which the period studied was from Days 31 to 40 of age. Following weaning the animals used in any particular experiment were placed into mixed-sex groups of six, same-aged, animals for the duration of the experiment. This allowed the experimenters to make detailed observations of the social behavior of the pups in the groups throughout this prepubertal period. Each group within each experiment was composed of a similar number of treated and untreated animals. This provided each animal with rearing companions of both sexes including normal, untreated animals.

### Experiment 1

In this first experiment the frequency of social play of intact male and female prepubescent rats was compared to that of male pups that had been castrated within 24 hr of birth. For while it had been found in an earlier experiment (Olioff and Stewart, 1978) that intact females exposed to TP injections on Days 1 and 2 of life engaged in more social play than did oiltreated females, the effects of removing the source of

T in males during the critical period for the organizational effects of T had not been studied.

## Methods

**Subjects** The animals used in this experiment were 16 male and 8 female Long–Evans hooded rats. The animals were selected from among the offspring of eight dams that were obtained while pregnant from the Canadian Breeding Farms and Laboratories, St. Constant, Quebec. Within 24 hr following birth the animals were removed from their mothers, sexed, and randomly assigned to treatment groups. Twelve males were castrated and twelve were sham operated. Surgery was performed under hypothermia anesthesia. The animals were then assigned to six mothers. Each mother received 10 pups (the average litter size for this species) 4 of which were similarly treated males and 6 of which were same-aged females. The animals were then left undisturbed until weaning. The animals had continuous access to food (Purina Lab Chow) and water. A 12L/12D light schedule was maintained in the animal colony (lights off at 0900 hr).

On Day 23 the animals were separated from their mothers and housed in four groups of six animals each. By this time five castrated males, one intact male, and one female had died; therefore, three of the four groups were composed of two castrated males, two intact males, and two same-aged females, while the fourth was composed of three intact males, one castrated male, and two females making for a total of seven castrated males, nine intact males, and eight females. The animals were maintained in these groups throughout the experiment. The animals were marked for identification (colored felt-tipped pens) approximately every 5 days and were not otherwise handled. Although they were housed in a different room, the animals were maintained on the same feeding conditions and the same light schedule as in the animal colony.

**Apparatus and Procedure** Each postweaning group was housed in a cage  $51 \times 33 \times 26$  cm, one wall of which was made of 1.25-cm plywood and had mounted on it a wire-mesh feeder and two water bottles. The remaining sides were made of 0.6-cm wire mesh.

The animals were observed daily during the prepubertal period between Days 26 and 40. Each group of six animals was observed for 70 observation periods per day. Each lasted for 20 sec, and during that period the behavior of all six animals was scored. If, during the period an animal engaged in a play-bout it was given a score of "1." Animals that did not engage in a play-bout were given a score of "0." Thus, for any animal the possible range of scores for each day of obser-

vations was from 0 to 70. An animal that engaged in any of the behavioral components of a play-fight sequence (see below) was considered to have engaged in a play-bout. All observations were conducted between 1200 and 1600 hr.

**Behavioral Definition of Social Play** Social play in rats is comprised of several individual behavioral components. While these components resemble in some respect those of agonistic encounters in adult rats, there are important distinguishing features. For instance, the distress vocalizations that are common to the agonistic encounters of adult rats are rarely heard during social play (Calhoun, 1962; Meaney and Stewart, 1981). Another feature of social play is that, unlike adult agonistic encounters, roles are frequently reversed; an animal that is dominated for a brief period during a play-bout will often immediately pounce on the other animal and then dominate it (see Poole and Fish, 1976).

The following is a description of the behavioral components of a playfighting sequence. *Pouncing*: One pup lunges at another with its forepaws extended outward. It is the forepaws that first make contact with the other animal. Pouncing is considered as a play-initiation act since animals exhibiting this behavior invariably engage in a play-bout so long as the recipient animal responds, and because it temporally precedes any other behavior in the play sequence. A complete play-fight sequence involves, in order, Pouncing, Wrestling, often Boxing and/or Lateral Display, and finally On-Top/On-the-Back Postures. In some bouts, however, animals withdraw prior to the On-Top/On-the-Back stage. (See Poole and Fish, 1976 for more information on behavioral transitions in play-fighting.) A play-fight sequence between pups 26 to 40 days of age usually lasts about 8 sec or less (Meaney and Stewart, in preparation). *Wrestling*: Two animals roll and tumble with one another. *Boxing*: Two animals standing upright facing one another and making pawing movements toward one another. *Lateral Display*: One animal arches its back and, with all four limbs extended, directs its flank toward another animal. *On-the-Back Posture*: One animal lies on its back fully exposing its ventral surface to another animal. *On-Top Posture*: One animal positioned over another animal with its forepaws placed on the other animal.

**Data Analysis** Total play scores were calculated for individual animals across all the observation days. The effects of the treatment conditions were studied by comparing the scores of animals in each of the three conditions regardless of observation group using a Kruskal–Wallis *H* test. Post hoc, paired comparisons were made using Mann–Whitney *U* tests.

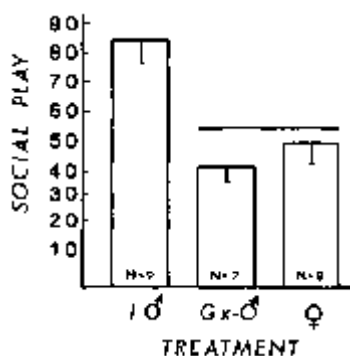


Figure 24.1

Mean ( $\pm$ SE) number of 20-sec sessions in which intact males (I-♂), Day 1-castrated males (Gx-♂), and intact females (♀) were observed to be engaged in play-fighting.

### Results and Discussion

The results of Experiment 1 are summarized in figure 24.1. Intact males were observed to engage in social play more than either intact females or castrated males. Statistical analysis revealed a significant treatment effect ( $H = 9.67$ ,  $P < 0.01$ ). Post hoc analysis showed that intact males engaged in significantly more social play than did either castrated males ( $U = 7$ ,  $P < 0.005$ ) or intact females ( $U = 8.5$ ,  $P < 0.005$ ). The difference between the castrated males and the intact females was not significant. These results indicate that genetic male rats deprived of testicular hormones from birth show greatly reduced levels of social play in the prepubertal period.

### Experiment 2

Since the castrated animals in the first experiment were deprived of testicular hormones from birth onward, it is not possible to specify the timing of the androgen effect. The results of Oloff and Stewart (1978) support the view that the androgen influence is limited to the neonatal period since females treated with TP, but which lacked, at least, the testicular source of androgens during the period of observation, did not differ from intact males in the frequency of social play. This suggests that there is no activational influence of circulating androgens on social play, and that the effect of testicular hormones is specific to the neonatal period.

Experiment 2 was designed to examine the possible effects of circulating androgens on the social play of male pups. In this experiment the frequency of social play of intact male and female pups was compared to that of males that were castrated on Day 23. Since the Day 23 castrates were without testes throughout the period of observation (Days 31 to 40), but not during the early neonatal period, it was possible to examine whether the presence of testicular hormones contrib-

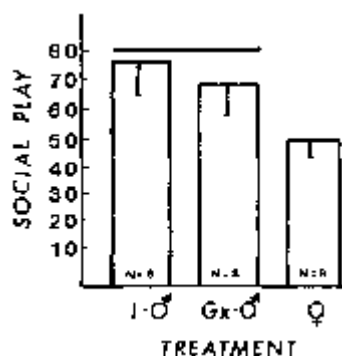


Figure 24.2

Mean ( $\pm$ SE) number of 20-sec sessions in which intact males (I-♂), Day 23-castrated males (Gx-♂), and intact females (♀) were observed to be engaged in play-fighting.

utes to the expression of typical levels of social play seen in male pups.

### Methods

**Subjects** The animals used in this experiment were 16 male and 8 female Long-Evans hooded rats. The animals were obtained and housed in the same way as in Experiment 1. On Day 23, 8 males were castrated and 8 were sham operated. Surgery was performed under Nembutal anesthesia. Following surgery the animals were placed into groups of six same-aged animals. There were four such groups each composed of two castrated males, two intact males, and two intact same-aged females.

**Apparatus and Procedure** The apparatus and procedure were the same as in Experiment 1, with the exception that the period of observation was shortened to 10 days (Days 31 to 40). This was done to allow for recovery from surgery.

**Data Analysis** The data from this experiment were treated similarly to those from Experiment 1. Note, however, that there were 10 and not 15 days of observation in this experiment.

### Results and Discussion

The results of Experiment 2 are summarized in figure 24.2. As can be seen both intact males and castrated males were observed to engage in more social play than did normal females. The statistical analysis across all three groups revealed a moderate treatment effect ( $H = 4.59$ ,  $0.10 > P > 0.05$ ). The post hoc analysis showed that both the intact males ( $U = 13.5$ ,  $P < 0.05$ ) and the castrated males ( $U = 17$ ,  $P < 0.06$ ) engaged in more social play than did the females. There was no significant difference between the two male groups.

The fact that castration at 23 days of age did not reduce the frequency of social play in prepubescent male rats suggests that there is no activational influence of circulating androgens on social play. Together with the findings of Experiment 1 and those of Oloff and Stewart (1978) these results indicate that exposure to testicular hormones in the early neonatal period is crucial for the development of the sex difference in the social play of rat pups, but that the presence of testicular hormones at the time of testing does not contribute to the expression of the sex differences. This conclusion has received further support from the results of subsequent work. In one study (Beatty, Dodge, Traylor, and Meaney, 1981) it was found that castration on neither Day 10 nor 20 affected the frequency of play-fighting in male pups. In a second study we (Meaney and Stewart, in preparation) found that while daily injections of 200  $\mu$ g of testosterone propionate to male pups between 26 and 40 days of age increased mounting behavior, it had no effect on the frequency of play-fighting.

### Experiment 3

The results of Experiments 1 and 2 suggest that the sex difference in the social play of prepubertal rats is due, at least in part, to the influence of neonatal testicular androgens. It is possible, however, that this sex difference may also be influenced by a suppressive effect of ovarian hormones either at the time of testing or earlier. In Experiment 3 we examined the influence of ovarian secretions on the social play of prepubertal female rats. In this experiment the frequency of social play of intact male and female pups was compared to that of female pups that were ovariectomized on Day 1 of life.

### Methods

**Subjects** The animals used in this experiment were 16 female and 8 male Long-Evans hooded rats. The animals were obtained and housed in the same way as in Experiment 1. Within 24 hr following birth the animals were removed from their mothers, sexed, and randomly assigned to treatment groups. Ten females were ovariectomized and ten were sham operated. Surgery was performed under hypothermia anesthesia. The animals were then reassigned to mothers as in Experiment 1.

On Day 23 the animals were separated into four groups of six animals. By this time two ovariectomized animals had died. Each group was comprised of two intact males, two intact females, and two ovariectomized females.

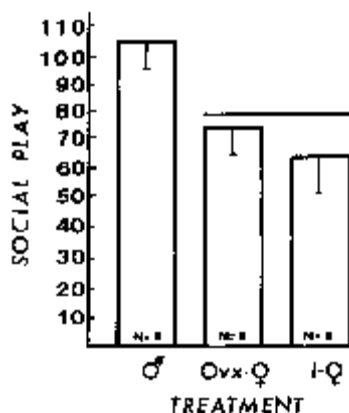


Figure 24.3

Mean ( $\pm$ SE) number of 20-sec sessions in which intact males (I-M), Day 1-ovariectomized females (Ovx-F), and intact females (I-F) were observed to be engaged in play-fighting.

**Apparatus and Procedure** The apparatus, procedure and analysis of the data were the same as in Experiment 1.

### Results and Discussion

The results of Experiment 3 are summarized in figure 24.3. As can be seen the intact males were observed to engage in more social play than did either of the two female groups. The statistical analysis across all three groups revealed a significant treatment effect ( $H = 6.76$ ,  $P < 0.05$ ). The post hoc analysis showed that the intact males were observed to engage in significantly more social play than did either the intact females ( $U = 10$ ,  $P < 0.01$ ) or the ovariectomized females ( $U = 16$ ,  $P < 0.05$ ). The difference between the two female groups was not significant.

These results indicate that there is no detectable influence of ovarian hormones on the social play of prepubertal female rats. Goy (1970) has reported that ovariectomy of female rhesus monkeys does not alter the frequency with which they engage in social play. Thus, in both the rhesus and the rat sex differences do not appear to be due to any suppressive effects of ovarian hormones on the social play of females.

### Experiment 4

The results of the first two experiments indicate that the sex difference in the social play of rat pups is dependent on the presence of testicular hormones during the early neonatal period. In this experiment we sought to examine whether T itself or one of its metabolites exerts this influence. It is known that within certain cells in the brain of the rat and of several other species, T can be converted either into  $E_2$  through the aromatization pathway (Naftolin, Ryan, and Petro, 1972) or into DHT through the 5 $\alpha$ -reductase pathway (Denef,

Magnus, and McEwen, 1974; Martini, 1978). Moreover, there are receptor sites, especially within the limbic system, for  $E_2$  (e.g., Stumpf, Sar, and Keefer, 1974), for DHT, and for T itself (Sar and Stumpf, 1974; Sheridan, 1979). It is conceivable then, that the testicular hormone effect described in Experiments 1 and 2 may be due to the action of T itself, to the action of T-derived  $E_2$ , or to the action of T-derived DHT.

In Experiment 4 the frequency of social play in genetic females treated neonatally (Days 1 and 2) with either TP, EB, or DHT was compared to that of normal male and female pups.

## Methods

**Subjects** The animals used in this experiment were 24 female and 12 male Long-Evans hooded rats. The animals were obtained and housed in the same way as in Experiment 1. On both Days 1 and 2 of life 6 females were injected subcutaneously with 250  $\mu$ g of TP, 6 with 250  $\mu$ g of DHT, 6 with 5  $\mu$ g of EB, and 6 with the oil vehicle alone. All steroids were in peanut oil solution and were delivered in 0.05-ml amounts. Collodion (Fisher Scientific Ltd.) was applied at the point of injection to prevent leakage. On Day 23 the animals were rehoused into six groups of six animals, each group containing 1 TP-treated female, 1 DHT-treated female, 1 EB-treated female, 1 oil-treated female, and 2 untreated, same aged males.

**Apparatus and Procedure** The apparatus, procedure, and analysis of the data were the same as in Experiment 1.

## Results and Discussion

The results of Experiment 4 are summarized in figure 24.4. As can be seen TP-treated females, DHT-treated females, and untreated males did not differ in the frequency with which they were observed to engage in social play, and all three groups were observed to engage in more social play than did both EB-treated females and oil-treated females. The statistical analysis revealed a significant treatment effect ( $H = 21.48$ ,  $P < 0.005$ ). Post hoc analysis showed that there was no significant difference between the males and either the TP-treated females or the DHT-treated females. Both TP-treated females and DHT-treated females were observed to engage in significantly more social play than did either the EB-treated females ( $U = 0$ ,  $P < 0.001$  and  $U = 5.5$ ,  $P < 0.03$ , respectively) or the oil-treated females ( $U = 0$ ,  $P < 0.001$  and  $U = 1.5$ ,  $P < 0.01$ , respectively). There were no significant differences between the EB-treated females and the oil-treated females.

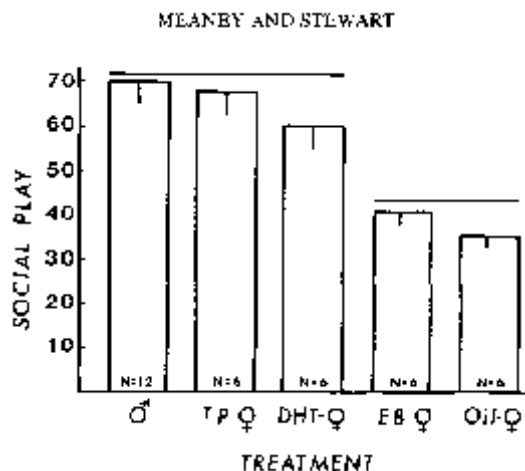


Figure 24.4

Mean ( $\pm$ SE) number of 20-sec sessions in which intact males ( $\sigma$ ), TP-treated females (TP- $\phi$ ), DHT-treated females (DHT- $\phi$ ), EB-treated females (EB- $\phi$ ), and oil-treated females (Oil- $\phi$ ) were observed to be engaged in play-fighting.

These results indicate that either T or its 5 $\alpha$ -reduced metabolite, DHT, administered during the early neonatal period is able to increase the frequency of social play in prepubescent female rats and, in the doses used, to eliminate the sex difference. In contrast, EB, even in doses sufficiently large to defeminize both open-field and lordotic behavior (see Stewart, Vallentyne, and Meaney, 1979), has no such effect. This suggests that the influence of T on the social play of rats is a true androgen effect.

## Experiment 5

The results of Experiment 4 suggest that the high levels of social play observed in male pups are due to the action of T or its DHT metabolite in the neonatal period, and that there is no effect of T-derived  $E_2$ . In the present study we tested this hypothesis using intact male rats. This was done by implanting newborn male pups with Silastic capsules containing either androst-1,4,6-triene-3,17-dione (ATD) or 4-androsten-3-one 17 $\beta$ -carboxylic acid (testosterone 17 $\beta$ -carboxylic acid or 17 $\beta$ -CA). ATD is an aromatase inhibitor and is presumed to limit the conversion of T to  $E_2$  (Leiberberg, Wallach, and McEwen, 1977). ATD has been shown to attenuate the  $E_2$ -mediated defeminizing effects on female sexual behavior (Booth, 1977; Clemens and Gladue, 1978; McEwen, Lieberberg, Chaptal, and Krey, 1977; Vreeburg, van der Vaart, and van der Schoot, 1977). In the present study the ATD capsules were left in the animals from Days 1 to 10. Thus, the endogenous levels of T-derived  $E_2$  should have been substantially reduced during the period when  $E_2$  normally acts to defeminize rat behavior. If T-derived  $E_2$

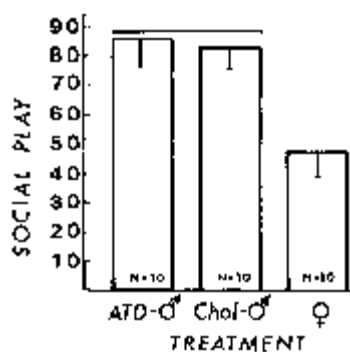
were involved in the development of male-typical levels of social play in the male rat, then ATD would be expected to reduce the observed frequency of social play. In the first part of this experiment (5A) the frequency of social play in normal male and female rats was compared to that of males implanted with ATD during the early neonatal period.

Similarly,  $17\beta$ -CA is a compound that inhibits the reduction of T into DHT (Kao & Weisz, 1979; Luttge, Jasper, Sheets, and Gray, 1978). In the second part of this experiment (5B) we implanted male pups with Silastic capsules containing  $17\beta$ -CA from Days 1 to 10, thus reducing the endogenous levels of T-derived DHT. If the metabolism of T into DHT is necessary for the development of male-typical levels of social play, then animals implanted with  $17\beta$ -CA during the neonatal period should differ from normal males in the frequency with which they engage in social play. In Experiment 5B we compared the frequency of social play in normal male and female pups to that of  $17\beta$ -CA-treated males.

## Methods

**Subjects** The animals used in this experiment were 36 male and 18 female Long-Evans hooded rats. The animals were obtained and housed in the same way as in Experiment 1. Within 24 hr following birth three groups of males were given Silastic implants containing either ATD ( $n = 10$ ), cholesterol (Chol;  $n = 18$ ), or  $17\beta$ -CA ( $n = 8$ ). The implants were inserted under the skin using hypothermia as anesthesia and removed on Day 11 using ether anesthesia. The implants were made from Silastic tubing 0.058 mm i. d. and 0.077 mm o. d. The 13-mm-long implants were filled with 7 mm of steroid and were sealed at each end with 3 mm of Silastic adhesive. The implants were then soaked in absolute alcohol for 1 hr to clean them and to check for leakage. They were then kept in 1% bovine serum albumin in PBS for at least 24 hr before use (see McEwen et al., 1977). In our laboratory we have used this procedure with ATD to prevent the defeminization of lordotic behavior in male rats (Stewart et al., 1979). At 23 days of age the animals in Experiment 5A were placed into five groups of six, each group composed of 2 ATD-treated males, 2 Chol-treated males, and 2 untreated, same-aged females. At the same age the animals in Experiment 5B were placed into four groups of six, each group composed of 2  $17\beta$ -CA-treated males, 2 Chol-treated males, and 2 untreated, same-aged females.

**Apparatus and Procedure** The apparatus, procedure, and analysis of data were the same as in Experiment 1.



**Figure 24.5**

Mean ( $\pm$ SE) number of 20-sec sessions in which cholesterol-treated males (Chol-♂), ATD-treated males (ATD-♂), and intact females (♀) were observed to be engaged in play-fighting.

## Results and Discussion

The results of Experiment 5A are summarized in figure 24.5. As can be seen, both ATD-treated males and Chol-treated males were observed to engage in more social play than did the females. The statistical analysis of the data revealed a significant treatment effect ( $H = 12.77$ ,  $P < 0.005$ ). Post hoc analysis confirmed that both ATD-treated males ( $U = 20$ ,  $P < 0.01$ ) and Chol-treated males ( $U = 19$ ,  $P < 0.01$ ) had significantly higher social play scores than did the females. There was no significant difference between the two male groups. There was, then, no effect of ATD treatment between Days 0 and 10 on the frequency with which male pups engaged in social play. It might be argued that after the removal of the ATD implants on Day 10, the resulting normal levels of T-derived  $E_2$  might be capable of masculinizing social play after Day 10. Recently, however, it has been found that the castration of male pups as early as Day 10 does not influence the frequency with which they engage in social play (see Beatty et al., 1981). Thus, in males the masculinization of social play occurs before Day 10 and, with respect to the present experiment, before that time when the implants were removed. The results of this experiment, then, extend and confirm the findings of Experiment 4 that in the the rat  $E_2$  in the immediate postnatal period neither promotes nor is necessary for the development of male-typical levels of social play.

The results of Experiment 5B are summarized in figure 24.6. As can be seen both  $17\beta$ -CA-treated males and Chol-treated males were observed to engage in more social play than did the females. The statistical analysis of the data revealed a significant treatment effect ( $H = 9.02$ ,  $P < 0.02$ ). Post hoc analysis showed that both  $17\beta$ -CA-treated males ( $U = 2$ ,  $P < 0.001$ ) and Chol-treated males ( $U = 14.5$ ,  $P < 0.04$ ) had significantly higher social play scores than did the females. There was no significant difference between the two male groups.

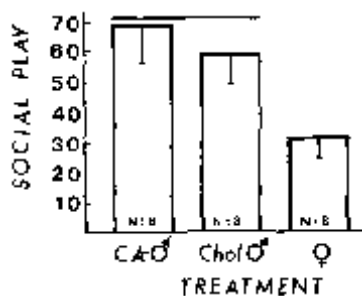


Figure 24.6

Mean ( $\pm$ SE) number of 20-sec sessions in which  $17\beta$ -CA-treated males (CA-3), cholesterol-treated males (Chol-3), and intact females (♀) were observed to be engaged in play-fighting.

The results of Experiment 5B would seem to indicate that the metabolism of T into DHT is not a necessary step for the androgenic effect on social play. This conclusion must be somewhat tentative, however, in that we have no direct measure of the degree of inhibition produced by  $17\beta$ -CA.

### General Discussion

Before beginning a discussion of these results it may be useful to consider briefly the nature of the sex difference in the play-fighting of prepubescent rats. The usual sequence involved in a play-fight between two young rats is that one animal approaches and pounces on another, wrestling and less often boxing ensues, and finally one animal emerges on top of the other (also see Poole and Fish, 1976). In animals younger than 40 to 45 days of age these bouts usually last 7–8 sec or less (Meaney and Stewart, in preparation; also see Poole and Fish, 1976). This sequence is the same for both male and female pups (Poole and Fish, 1976). One difference in the pattern between males and females is that females tend to withdraw sooner from play bouts than do males (Poole and Fish, 1976; Meaney and Stewart, in preparation). Thus, Meaney and Stewart (1981) found that males engaged in more On-Top Posture than did females. The single feature that contributes most to the sex difference in play-fighting, however, is that of Pouncing or play-initiation; in addition males initiate play more often with males than with females (Meaney and Stewart, 1981). Thus while male rats are more playful than females (i.e., males initiate and become involved in more play-fights) the basic patterns of play-fighting of males and females are similar (see also Poole and Fish, 1976).

The results of these experiments support the view that the sex difference in the social play of prepubescent rats is an androgen-mediated effect, dependent on neonatal exposure to either T or DHT. The  $E_2$  metabolite of T does not appear to be directly involved in the

processes that result in the development of male-typical levels of social play.

The present experiments do not provide information about precisely how long into the neonatal period T or DHT may be effective in influencing social play, but the results of Experiment 4 do indicate that exposure to TP or to DHT on Days 1 and 2 of life is sufficient to masculinize female rats. In addition, the results of Experiments 2 and 4 demonstrate that circulating testicular androgens are not necessary for the expression of male-typical levels of social play. Neither males castrated at 23 days of age nor females given TP neonatally, both of which lacked testicular androgens at the time of observation, differed from intact males in the frequency with which they engaged in social play. Taken together these findings indicate that, while testicular androgens are not directly responsible for the expression of male-typical levels of social play, their activity during the neonatal period contributes to the sexual differentiation of some CNS function that in turn mediates this behavioral sex difference.

It is possible that this androgen-related sexual differentiation is mediated by androgen-receptor proteins in the CNS, most of which bind with both T and DHT (Sar and Stumpf, 1974). Androgen receptors have been detected in the limbic brain of the rat by postnatal Day 1 (Fox et al., 1978; Lieberberg et al., 1978). Moreover, these neonatal androgen receptors exhibit properties typical of androgen receptors in adult rats; most notably they bind to DNA cellulose with the same affinity (Fox et al., 1978; Lieberberg et al., 1978). This characteristic is important because the intracellular effects and in particular the growth-related effects of steroid hormones are mediated by DNA (e.g., Salaman and Birkell, 1977). Thus, one possible explanation for the masculinizing influences of neonatal androgens on the social play of rats is that the exposure to androgens during the neonatal period promotes the formation of sex-specific neural circuitry and that this circuitry serves to mediate the sex difference in social play that is seen in prepubescent animals.

Recently we (Stewart et al., 1979) have reported a role for the androgenic actions of T in neonatal rats in the establishment of the sex difference in open-field behavior seen in adult animals. The sex difference in open-field behavior, like that in social play, is not dependent on the presence of gonadal hormones at the time of testing (Bengelloun, Nelson, Zent, and Beatty, 1976; Blizard, Lippman, and Chen, 1975; Bronstein and Hirsch, 1974; Stewart and Cygan, 1980) although it is dependent on the presence of androgens in the neonatal period (Blizard and Denef, 1973; Gray, Lean, and Keynes, 1969; Pfaff and Zigmond, 1971; Stewart, Skvarenina, and Pottier, 1975). Female rats injected

on Days 1 and 2 with DHT and males treated neonatally with ATD, when tested as adults, exhibited male-typical levels of open-field activity (Stewart et al., 1979). Thus, both social play and open-field behavior can be masculinized through the neonatal exposure to the androgenic component of T. These findings, taken together with the evidence that the androgenic actions of T play a role in the perinatal period in the development of adequate male sexual behavior (Clemens, Gladue, and Coniglio, 1978; Davis, Chaptal, and McEwen, 1979; Nadler, 1969; Stewart and Kaczender-Henrik, 1971; Ward and Renz, 1972), strongly suggest that there is a role for neonatal androgen receptors in the development of male-typical behavior.

Finally, it is interesting to note that these androgenic effects on the behavior of the rat closely parallel those found in the rhesus monkey. Goy (1978) has found that prenatal exposure to either T or DHT acts to masculinize both prepubertal play and male copulatory behavior in female rhesuses. This suggests that the masculinization process in the rat is comparable to that of this primate species.

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The previous chapters hypothesized that “neural tissues” are organized or affected by hormone action during development and that it would be this neural circuitry that would, in turn, affect the behavioral dimorphisms. To this point in the reader, however, we have not read any experiments designed to demonstrate a neuroanatomical difference between female and male linked to the dimorphic behaviors described. This section contains studies designed to demonstrate differences between female and male central nervous system structures that are, strongly or not, purported to be associated with sexually dimorphic behaviors. Experimental models range from birds to rats to primates, including humans. This section contains some elegant and simple experiments as well as some elegant and extremely complicated experiments. The experiments delve more deeply into the role steroid hormones play in the development and maintenance of nervous system structures. In some cases, the dimorphisms exist simply because androgens are responsible for maintaining structures that are present in both XX and XY organisms at birth but which degenerate in females due to the lack of androgens.

Ask yourself which models you feel best demonstrate sexual dimorphisms. Do all behaviors and species have their own dimorphisms, or are some behaviors and/or species more likely to be dimorphic? Do all anatomical differences have strong behavioral correlates? If they don't, does this undermine the importance of the morphological dimorphism? What should be the normative standard against which a difference is measured? And, is bigger better?

Reading the chapters in this section will provide a familiarity with some of the most obvious morphological dimorphisms of the vertebrate nervous system in order to

1. Further interrogate the concept of sexual dimorphism;
2. Raise the question of: when does a difference make a difference?;
3. Suggest that functional differences may be studied more appropriately on a synaptic level;

4. Demonstrate that the most important differences may not be in numbers or density of neuron but in the circuits they form and the neurotransmitter systems that innervate them; and
5. Suggest that most differences have been determined using the male model as the norm.

### The Song Bird

The song bird (zebra finch and canary) has one of the strongest sexual dimorphisms in all the literature on sex differences. Males sing and females do not really. It is a behavioral difference that depends on structures in the bird's brain that are also very evidently different between the sexes—remove or prevent the development of the nucleus, and the male bird either does not sing or his song is drastically diminished. This functional/structural difference is under hormonal control but, interestingly, social experience and training contribute. Without “tutoring” by an older male, young birds do not develop a very complicated song. So, this is a lovely system in which to study the interaction of nature and nurture in the development of a difference that makes a difference.

The Nottebohm and Arnold chapter is a review that lays out the work done in Nottebohm's lab prior to 1976. It describes the anatomy of the songbird's brain and identifies the structures involved in song production. It also demonstrates which neural structures involved in song production are different between the sexes, which are not and by how much.

The Nottebohm chapter demonstrates that testosterone presence or administration leads to the development of the dimorphism in song nuclei and, when administered to female canaries, triggers the growth of the song nuclei to rival those of the male. This growth in the nuclei is accompanied by the development of song in the female. The experiments reported here also determine that this increase in the size of the nuclei can be affected in the adult bird and is unusual in that respect, presaging a later understanding that in some birds the nuclei wax and wane with the mating season.

Finally, if a male bird sings in the forest and the female has no song nuclei, will they mate?

The Brenowitz chapter starts with the question of why, if the female doesn't produce song, does she have song nuclei, small though they may be? This is a great question, bypassing the notion that size is everything. It leads to the understanding that, at least in the canary, one of the nuclei, the nucleus of the ventral hyperstriatum (HVC), is responsible in the female for the *recognition* of species-specific song, thereby being of critical importance in the same behavior that the nucleus serves in the male, successful mating.

Read these chapters for the form of experimental design and the ability to closely tie a complex behavior with neural structures. Note how the design builds on the mammalian experiments of Beach and of Young with androgens or estrogens being administered to determine whether the behavior depends somehow on steroid hormones and whether by steroid hormone administration, a male behavior can be induced in females. Read these papers also for the methods used to tease apart a complex system of brain structures that all contribute to a given behavior—it is not as simple as ONOB, one nucleus, one behavior. Note also that the female is not treated as a passive, default system but one that has to undergo a particular type of development for their role—listening and recognizing. Without the female behavior, the male behavior will not work.

### The Mammalian Spinal Cord

The mammalian spinal cord provides another strong example of a difference in neural structure that can be linked with a difference in behavior, the spinal nucleus of the bulbocavernosus (SNB in rodents, Onuf is nucleus in humans). The SNB is a group of motor neurons whose axons innervate muscles associated with the perineal striated muscles that mediate penile erection in males and aspects of orgasm in females: the bulbocavernosus and levator ani muscles. These muscles are present in male and absent in female rodents. Interestingly, when the SNB is compared across species, in some species there is very little difference between females and males. Thus, the number of motor neurons is small to nonexistent in female rodents where the muscles are nonexistent and obvious to visual inspection when Nissl stained in the male, comprising large neuronal cell bodies. In dogs and humans, however, the muscles exist in the female, thus the motor neurons exist in the female. Roughly speaking, the dimorphism decreases as the phylogenetic scale is climbed with it being the least different between the sexes in humans.

In the first chapter, Breedlove and Arnold demonstrate that the motor nucleus exists and is dimorphic.

As well, they demonstrate that the motor neurons accumulate radioactively labeled androgens but not estrogens and that the nucleus depends on hormonal action and not on genetic sex, because the nucleus is absent in both the female and male in animals that do not make the androgen receptor.

In the second chapter, Breedlove and Arnold take these observations further to demonstrate that normal male rodents treated with an androgen blocker, flutamide, do not develop the nucleus and, in that sense, resemble females. This also supports the interpretation of the previous chapter, that the development of the nucleus depends on the presence of androgens. Interestingly, the results of this experiment support an understanding that sex is complicated: Breedlove and Arnold can eliminate the motor neurons responsible for erection but the fact that the male cannot achieve intromission does not affect mounting and other copulatory behaviors. As in the Young and the Meaney chapters, when different amounts of androgens are blocked or administered during development, the nucleus is affected to a greater or lesser extent. With no circulating testosterone, the males not only fail to develop the spinal cord nucleus but the target muscles as well, indicating the developmental dependence of targets on their inputs and vice versa.

Forger and Breedlove carry the studies further by comparing the nucleus in the dog and human spinal cords, again, studying the role of androgens during a critical period for sexual development. They find that in female dogs, as in rodents, the dimorphism can be eliminated by administration of androgens, which serves to maintain both the muscles and the motor neurons that innervate them in the female dog.

Read these chapters for the strength of the dimorphism they describe, the role of androgens in its development, and the quantitative differences as they are manifest in different species. Ask yourself whether a sexual dimorphism is likely to be stronger if it is associated with sex? Because there is only slight difference between human females and males for spinal nucleus dimorphism, it is tempting to speculate that human sexual function is less different between females and males than is rat sexual function and that clitoral excitation in females may require the presence of these muscles as does penile excitation. Interestingly, these muscles may be cut in the traditional practice of female genital circumcision (or, mutilation) and it is worthwhile speculating on what effect this might have on Onuf's nucleus. It is worthwhile wondering at this point whether the decrease in dimorphism of this motor nucleus the higher one goes on the phylogenetic scale will also be the case for other central nervous system dimorphisms.

### The Mammalian Brain

Understanding sexual dimorphisms of the mammalian brain naturally led to studying the neural circuits involved in hormonal release and the ovulatory cycle. In fact, when one is considering organizational effects, it seems that one of the most fundamental neural organizations would be whether neural circuits are established in such a way as to produce a monthly surge of luteinizing hormone. This is one of the clearest examples of a function related to sexuality that females have and males do not with the concomitant expectation that the circuitry necessary be present in females and absent in males. Because an absence of function has generally been interpreted as negative or “less than,” it is a relief to look at studies in which the presence of, more than, and bigger are being investigated in females rather than in males. But, perhaps more important, one of the results of these studies is that the circuitry for hormonal cycling is present in primate males as well as females and that it is “activated” in females but not in males.

Raisman and Field’s study on this topic is unsurpassed in its thoroughness, thought, and insight. To this day, it remains an example of how studying sexual dimorphisms should be done—with caution and an open mind. The authors take the imaginative leap of designating their groups, not by chromosomal sex, but by function: that is, by whether the treatment group has a hormonal cycle. They then study the neuronal circuitry in an area of the brain identified as a region upon which cycling depends, a region between the ventral medial and arcuate nuclei of the preoptic region of the hypothalamus. Step by step, they tease apart the synaptic circuitry in this brain region even though at the time of this experiment methods of identifying the origin and type of synaptic input were quite limited. Raisman and Field take advantage of every means available to them using anterograde degeneration and scrupulous descriptions of the fine structure of synaptic terminals. While the descriptions of synaptic morphology may seem a bit arcane, the authors are able to differentiate enough of the input to determine that cycling rodents (male and female) have a higher density of nonamygdaloid (not from the amygdala) synapses on dendritic spines than noncycling animals. And that this difference can be induced by the elimination of androgens by castration at 12 hours but not 7 days postnatal as well as erased by the administration of testosterone by day 4 postnatal.

In the next chapter Allen and Gorski follow on this understanding and report a sex difference in the bed nucleus of the stria terminalis of the human brain; remember that Raisman and Field’s work focused on the synapses of axons passing through the stria termi-

nalis. Thus, Allen and Gorski are trying to understand this dimorphism in the human. Given their methods, all Allen and Gorski, two groundbreaking neuroscientists in the field of sexual dimorphisms, can say is that there is a difference, but they use this difference to suggest that the human brain, like the rodent, establishes these sex differences by its sensitivity to circulating steroid hormones during a developmentally sensitive period. In this case, the volume of the darkly staining region of the bed nucleus is greater by volume in males than females.

Read these chapters for the detail of description and the focus on a uniquely female function. Through no fault of the authors, note the paucity of detail in the human studies as compared to the experimental animals. This is a theme that will reoccur—because it is difficult to work with postmortem material, which by necessity, all human anatomical experiments before in vivo imaging required. Compare the quantitative opportunities available to understanding differences in counting types of synapses as opposed to measuring the volume of a darkly stained region. Consider the difficulty involved in delimiting the area to study in Nissl staining of the hypothalamus and the possible variation in life experience from human to human.

### The Anatomy of Difference

On the heels of brain differences related to cyclic hormonal release and the anatomical differences in the male and female brain regions devoted to song in the songbird, comes the quest for regions of the male mammalian brain that are preferentially represented because of the organizing action of androgens during development. The theme emerges that because they depend on the presence of androgens, these are regions of male brains involved exclusively in male sexuality. Underlying this thinking is the assumption that, if they are related exclusively to male sexuality, they will be larger in males.

In the first selection within this chapter, Gorski and his colleagues describe a region of the medial preoptic area of the hypothalamus that is significantly larger in the male rat than in the female. Searching in the preoptic area of the hypothalamus because it is a region known to be affected by the organizing effects of androgens, they identify a cell-dense region larger in males than in females that they call, the sexually dimorphic nucleus of the medial preoptic area or, the SDN of the MPOA. Although no known function was associated with it when it was first described, the difference was so robust—the difference in size between females and males can be discerned by the naked eye—that in the absence of any known function, the SDN becomes the anatomical difference that has been

most studied with respect to behavioral differences. In this selection, Gorski and his colleagues demonstrate that not only does this region exist but that its size is indeed under the organizational control of androgens. In subsequent works, Gorski and his colleagues carefully tease apart size and cell density using blinded observations and inter-reader reliability methods for establishing the boundaries of this cell group, painstakingly counting neurons in order to establish a difference that makes a difference.

Subsequently, Swaab and Fliers study human autopsy material and report on a cell grouping in the homologous region of the human brain, that by volume is 2.5 times larger in males than females. In keeping with the terminology used by Gorski, they call it the “sexually dimorphic nucleus,” claiming it to be the human equivalent of the nucleus described by Gorski and colleagues. As the first team to make such a report for the human brain, their paper is an important landmark in the continuing study of dimorphisms.

Allen and Gorski follow their own report on the rodent and Swaab’s report on humans with another report on the human brain searching again for a homologous difference in humans which they call the “preoptic-anterior hypothalamic area” (PO-AHA). In contrast to Swaab and Fliers’ study, Allen and Gorski’s study reveals four regions, which they name the “interstitial nucleus of the anterior hypothalamus 1–4” (INAH 1–4). In their hands, INAH 2 and 3 are sexually dimorphic, and 1 and 4 are not. This study introduces an interesting twist on the search for sexual dimorphisms in humans in that they suggest that what Swaab and Fliers called the SDN is in fact by placement, cell density, and relationship to other hypothalamic structures, what Allen and colleagues refer to as the INAH 1, which in their material is not dimorphic. Thus begins a controversy that brings into the quest the reliability of human postmortem material, methods for demarcating the boundaries of nuclei in a region such as the hypothalamus, where there is no clear laminar structure and the confounding factors of human lives such as individual experiences, nutrition, aging, and hormonal action throughout a long and varied lifespan.

Read these selections for the methods used and compare them to the methods used by Raisman and Field. Ask yourself whether the difference in the detail of the information gleaned supports the relative efforts of each study. Note the painstaking identification of the relative regions of the hypothalamus through multiple planes of tissue sections and the difficulty of discerning the boundaries of nuclei in three dimensions in a region with such diffuse cell groupings—notice that it is difficult in the rodent but even more difficult in the human.

Consider the difficulty of working with human tissue—getting enough in the same age groupings (usually a range of 10 years) for statistically significant results and ask yourself how individual experience/differences in life’s circumstances might continue to shape regions of the human brain. Can we justify our sense of differences between humans with studies that show enormous variations between cases? Are the differences within a category (i.e., between women) as great as the differences between the categories (i.e., between women and men)?

### Physiological Correlates

What does a difference between structures mean if the structure is not correlated with a function?

The selections in this chapter detail the search for a function for the SDN-POA in both rodents and primates. Because differences between anterior hypothalamic areas in rodent and humans were described before there were known functions for any of these regions, these functional studies were conducted post hoc with an eye toward finding a sexual function. The experiments described here were remarkably difficult to carry out, in some cases requiring behavior in animals with implanted electrodes and complicated restraining devices. These studies use three different methods for studying function: (1) destroying a region and looking for an absence of function; (2) electrically stimulating a region and observing what behavior it incurs; (3) recording electrical activity as a behavior takes place.

These are astonishing experiments that require assumptions about human and animal behavior that are not so easy to corroborate. Because the findings are disparate the studies may seem uninformative. These studies are worth exploring, however, if only to gain perspective on how difficult it has been to identify precisely how a sexually dimorphic nucleus relates to sex. They also reveal a fascinating disjunction between different aspects of sexuality. Sex might feel like a unified act but just as vision and memory comprise facets of neuronal activity that are somehow bound into a singular experience, sex is also not unitary.

Arendash and Gorski’s contribution is a report on their attempt to understand what role, if any, the SDN-POA plays in the expression of male sexual behavior. To do this, they placed relatively circumscribed lesions in the SDN-POA, dorsal MPOA or ventral MPOA of adult male rats. They then observe what aspects of male sexual behavior are absent in those animals with lesions, finding that lesions in the dorsal MPOA disrupt the number of mounts, intromissions, and ejaculations. They also find that lesions to the SDN-POA alone have no effect on male copulatory be-

havior. They conclude that *if* this region that is significantly larger in males than females plays a role in male sexual behavior, it must be a subtle one.

Perachio and colleagues take the experiment to primates and search for the function of various hypothalamic regions by stimulation. In Rhesus monkeys they implant electrodes in the preoptic area, lateral hypothalamus, and dorsomedial nucleus of the hypothalamus (DMH). They find that in the presence of receptive females, stimulation of the DMH leads to longer mounts with more thrusts per mount, more ejaculations, and shorter refractory periods between ejaculations. Preoptic stimulation however, decreases the thrusting rate and eliminates ejaculation. It is interesting, however, that stimulation of the anterior hypothalamus leads to erection in socially isolated males, while stimulation in the presence of a female does not lead to erection. This supports the contention that the preoptic area plays a role in sexual behavior but in this case, stimulation of the region seems to be associated with a *decrease*.

The Oomura experiment is extraordinary, and one only wishes there were a figure of the apparatus! Oomura and colleagues record from different regions of the anterior hypothalamus of male Rhesus monkeys to understand how neurons in these regions fire preceding, during, and after sexual activity with females. The results of this experiment reveal that neurons in some regions fire before sexual activity, indicating desire. Others fire during and are silent before and after. Still others fire at the end of ejaculation and are silent during intromission and ejaculation. They demonstrate that, at least in the male, different aspects of sexual activity are mediated by different brain regions. And they presage experiments in which sexual activity in human primates is studied by functional magnetic resonance imaging. At the very least it suggests that male sexual activity is not impeded by what might be considered fairly cumbersome techniques.

Read these selections for their creativity in experimental design as well as the precision of their measurements of animal behavior. Note the difference between what methods measuring the absence of behavior (brain lesion) and methods measuring behavior in situ (electrophysiological recordings) can reveal. Consider the surprising findings that a region that is larger in males cannot be found to play an active role in sexual behavior. Ask yourself how you would design experiments to determine the role of the SDN-POA in female sexual behavior in light of the fact that none of these experiments were carried out in females.

In sum, these studies reveal, at least in males, a stunning modularity to sexual function, which of perhaps of all the behaviors that humans practise is the one

that *feels* the most unified. And yet, when studied neuron by neuron it is as divisible as memory with some regions mediating desire, others intromission, and still others, ejaculation. In addition, although all these studies implicate the preoptic area in male sexual behavior, they find that the region that looks the most different between males and females does not actually mediate anything different that we can *measure*. This puts an interesting twist on the relation between a size difference and behavior, raising the question of what a size difference means. What may be true for the canary is not true for the rodents or primates.

### Neurotransmitter Systems

Studying neurotransmitter systems is a way of studying synaptic input with some aspect of the input identified. It has the added advantage of suggesting function since different neurotransmitter systems can be loosely associated with function. For example, with the advent of the selective serotonin reuptake inhibitors (SSRIs) as medications for depression, serotonin has been identified with mood as well as with attention and wakefulness. Thus, when higher densities or different patterns of innervation are found in females than males, there is a hint at a system that might require a different balance for mood stability than for reproductive function—with the balance between the two a delicate one.

The experiment by Simerly and colleagues elegantly demonstrates three regions of the medial preoptic nucleus (MPON) on the basis of differential staining with an antibody to serotonin. It reveals that the central region of the MPON (the SDN) is devoid of serotonergic fibers and that the pattern of staining around differing between females and males; because the SDN is smaller in females than in males, females have a more even distribution of serotonergic fibers and synapses with a smaller gap at the region of the SDN.

In the second selection of Simerly's he carries out the now classic experiment—depriving males of androgens or estrogens and inducing the female pattern of staining and of giving females androgens or estrogens to induce the male pattern of staining. This experimental paradigm solidifies this sexual dimorphism in neurotransmitter systems.

De Vries and colleagues' study identifies other neurotransmitter systems that differ between females and males suggesting that these differences may arise during the critical period for sexual differentiation. They report on their vasopressin studies and their use of immunocytochemistry to demonstrate that the distribution of vasopressin fibers in the lateral septum and the lateral habenular nucleus are denser in males than

in females. The experiments also identify a group of vasopressin-positive cell bodies in the bed nucleus of the stria terminalis that may be the origin of the vasopressin-positive fibers. Thus, while females have a higher density of non-amygdaloid synapses on dendritic spines in regions that receive input from the stria terminalis, males have a higher density of vasopressin-positive neurons that originate in the bed nucleus of the stria terminalis.

Read these papers for the way in which they build on the now classic paradigm of (1) identifying an anatomical dimorphism and (2) depriving or administering androgens/estrogens during early development to determine whether steroid hormones organize the dimorphism. Compare the different anatomical methods you have now read about used to identify sex differences—studying the ultrastructure of a particular region to determine synaptic differences, measuring the volume and/or counting neurons to determine greater or lesser size/neuronal density, or labeling a neurochemical system to determine its relative synaptic contribution. What do they tell you about function? At what level do you believe that differences that make a difference will be discernable? How would you use these studies to better understand why women have a higher incidence of depression than men? Would these studies have any relevance when treating conditions of the nervous system?

In many species of animals, males and females exhibit different patterns of behavior, especially in contexts related to courtship and reproduction (1). Recent evidence suggests that structural differences in male and female central nervous systems may contribute to these differences in behavior (2). We have discovered a striking sexual dimorphism in song control areas of the brain of the canary (*Serinus canarius*) and the zebra finch (*Poephila guttata*), which can be related to behavioral differences between the two sexes.

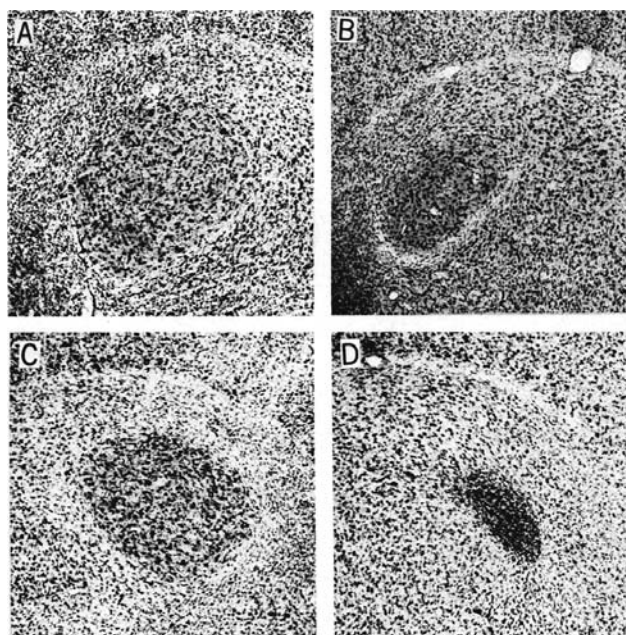
Adult male canaries have a complex song repertoire learned by reference to auditory information (3, 4). Female canaries do not normally sing, although they will produce a song similar to that of the males when administered testosterone (5, 6); the song, however, is considerably less varied than that of males (6). Female canaries also produce a variety of other calls (7), and, as in the case of other carduelines (8), some of these calls may be learned.

Male zebra finches have a single song type, which, as in the canary, is developed by reference to auditory information (9). Female zebra finches do not sing even when testosterone propionate is implanted when they are adults (10). The vocal repertoire of female zebra finches is otherwise small, consisting of contact and enticement notes produced in a variety of circumstances (11). Because of this simplicity, the calls may develop normally in the absence of auditory models.

Five adult male canaries, five adult female canaries, six adult male zebra finches, and seven adult female zebra finches (12) were anesthetized and then perfused with saline followed by 10 percent formalin in physiological saline. The brains were removed, blocked, fixed, embedded, and sectioned; the sections were mounted on glass slides (13, 14). All brains were weighed before being embedded (15). Serial sections cut at 10 to 50  $\mu$ m were mounted, stained with cresyl violet for cell bodies or silver stain (Fink-Schneider) (14) for unmyelinated nerve fibers, and viewed under the light microscope. The volume of certain brain structures was measured as follows. A microprojector (Bausch and Lomb) projected magnified ( $\times 53$ ) images of cresyl violet-stained sections on drawing paper. A perimeter was then drawn

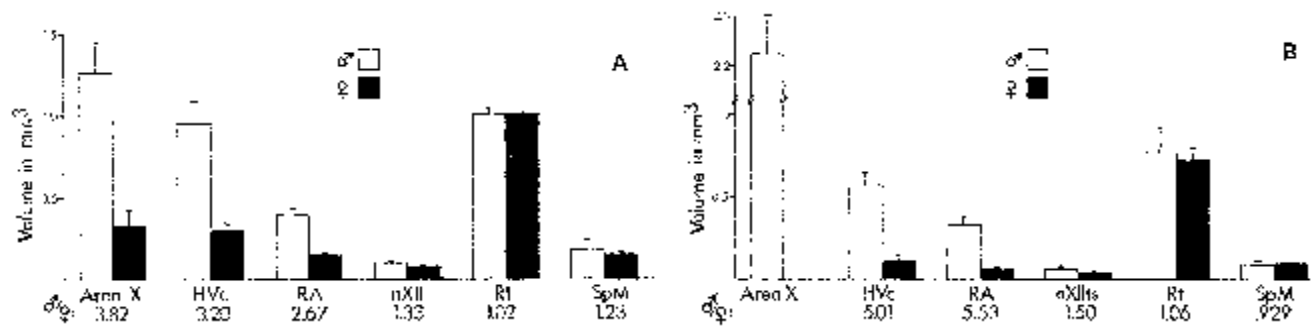
around the regions of interest, and the area enclosed was measured with a polar planimeter. These areas were then multiplied by the thickness of the sections, and the resulting volume was corrected for the frequency of sampling (for example, multiplied by 2 if every other section was measured). The sum of all such products for a given brain region was an estimate of its volume.

We made drawings of four cytoarchitectonically distinct brain structures: (i) area X of the lobus parolfactorius (LPO), (ii) the hyperstriatum ventrale, pars caudale (HVC), (iii) the robust nucleus of the archistriatum (RA) (figure 25.1), and (iv) the hypoglossal



**Figure 25.1**

Frontal sections through the robust nucleus of the archistriatum (RA) in a male (A) and female canary (B) and a male (C) and female zebra finch (D). The canary photographs are from the left hemisphere, and those of zebra finch are from the right. For each of the four birds shown, the rostro-caudal level corresponds to the largest area of RA seen in this plane of section. The relatively unstained eyebrow-shaped structure is the lamina archistriatalis dorsalis, which separates the neostriatum (dorsal) from the archistriatum (ventral). The prominent ellipsoidal nucleus is RA. Cresyl violet-stained sections, 50  $\mu$ m thick ( $\times 42$ ).



**Figure 25.2**

The volumes occupied by four neural regions associated with vocal behavior (area X, HVC, RA, and nXII) and by two regions not associated with vocalization (Rt and SpM) in male and female canaries (A) and zebra finches (B). Each bar represents the mean of the total (right plus left) volumes of each area sampled (18, 26), and the vertical line above the bar is the standard deviation of the individual values. The ratio of the male to the female mean is given for each region.

nucleus of the medulla (nXII) (16). Nottebohm et al. (4) have described these structures in the canary and presented anatomical and behavioral evidence that they are part of the vocal control system of the canary brain. The brain of the male zebra finch includes areas that are similar to these four regions in position and cytoarchitecture; we thus presume that they have a similar role in vocalization (17, 18). We also drew and estimated the volume of two thalamic brain structures not related to vocal control, the nucleus rotundus (Rt) and the nucleus spiriformis medialis (SpM), which were chosen because of their discrete boundaries (19).

In both the canary and the zebra finch, the four vocal control areas are markedly larger in males than in females ( $P < .02$ , two-tailed  $t$ -test) (figures 25.1 and 25.2). These differences are highly significant. That there were no such differences in the volume estimates of the two structures not related to vocal control or in total brain weight ( $P > .2$ ) (15) suggests that the differences are specific to song areas and related to a sexual difference in vocal behavior. This conclusion is also supported by the observation that the sexual differences in volume are more marked in zebra finches than in canaries, as would be expected from the total absence of song in female zebra finches.

In zebra finches, we detected no differences between the right and left sides of any of the four vocal and two nonvocal areas. Similarly, there was no systematic difference between the volumes of the right and left area X, HVC, RA, Rt, and SpM in canaries. This result is intriguing in light of the marked left hemispheric dominance for vocal control described for this species (4, 20). There was significant difference between the volumes of the right and the left hypoglossal nuclei of canaries (21). The larger size of the left side in all ten male and female canaries may be related to the left hypoglossal dominance for song control in this species (22).

The size ratio of male to female canary brain areas increases as one goes from the hypoglossal nucleus to RA, HVC, and area X, that is, as one goes to structures further removed from the motoneurons innervating the syringeal musculature (23). This graded series of ratios suggests that the “higher” (that is, further removed) neural regions in this system are involved in some aspect of vocal performance in males that is specifically absent or underrepresented in females. It may be that the higher centers are responsible for a disproportionately large share of the neural operations controlling vocal learning and size of vocal repertoire; therefore, in females, who normally do not sing, these areas should be less well represented. The zebra finch brain departs from this pattern in that the volume ratio of male to female brains is somewhat smaller in HVC than in RA (figure 25.2), and area X is not recognizable in the female.

In both male and female canaries, area X contains larger cell clusters than the surrounding LPO; perhaps as a result, cresyl violet stains area X darker than the surrounding tissue (4). In Fink-Schneider stains of unmyelinated fibers, area X is discriminable from the rest of LPO because it contains a rich mesh of fibers, some of which are projections from HVC (24). Area X in male zebra finches is similar in these respects [see also (19)]. However, in the corresponding area of the female zebra finch brain area X is not recognizable, which suggests that it is grossly modified or absent. We have assumed it to be absent (figure 25.2). It is not clear how the disproportionately large size of area X in the male zebra finch compared with that of the canary might relate to differences in behavior.

From the extent of the sexual differences in the volumes of the vocal areas, we infer that sexual differences are not confined to cell size alone (figure 25.1). The density of cell packing and the amount of neuropil in RA and HVC also differ between males and females

(25). Males, with a greater commitment to vocal learning, also have more neuropil. The sexual dimorphism in vocal areas of these two songbird species may be related to the fact that, whereas males of both species learn their song by reference to auditory information, females do not normally sing.

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10. On occasion an intact adult female that has had a pellet of testosterone propionate implanted will produce a repetitive vocalization not otherwise heard from members of this species. The manner of delivery of such a sound is reminiscent of song but its physical characteristics differ greatly from normal song [A. P. Arnold, thesis, Rockefeller University (1974)].
11. K. Immelmann, *Zool. Jahrb. Abt. Allg. Zool. Physiol.* 90, 1 (1962).
12. Wasserschlager canaries of our own inbred stock ranged in age from 19 to 34 months old. All but two of the zebra finches were descendants of a single pair of domesticated birds and were between 29 and 56 months (usually 29 to 31 months) old.
13. T. M. Stokes, C. M. Leonard, F. Nottebohm, *J. Comp. Neurol.* 156, 337 (1974). The method was modified in some cases. All canary brains were embedded in gelatin albumin and stained with cresyl violet. They were sectioned serially at 25 or 50  $\mu\text{m}$ , and drawings were made of neuronal regions in single sections at intervals of 100 and 125  $\mu\text{m}$ . All drawings of canary brains were made without prior knowledge of the sex of the animal. Of the zebra finch brains, five male and six female brains were embedded in gelatin albumin, sectioned serially at 25 or 50  $\mu\text{m}$ , and stained with cresyl violet. Drawings were made from sections taken at intervals of 50 to 100  $\mu\text{m}$ . One male and one female zebra finch brain were embedded in paraffin, sectioned serially at 10  $\mu\text{m}$ , and stained with cresyl violet. Of the gelatin albumin-embedded brains of zebra finches, one male and one female brain were sectioned serially at 25, 50, 25, 50  $\mu\text{m}$ , etc., and the 25- $\mu\text{m}$  sections were stained for unmyelinated fibers by the Fink-Schneider method (14).
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15. Canary brains were weighed after being fixed in sucrose-formalin, and zebra finch brains were weighed after being fixed in formalin. There was no statistical difference between the two sexes of either species in brain weights (two-tailed *t*-test,  $P > .2$ ). Means and standard deviations of brain weights were  $0.74 \pm 0.09$  g (male canary),  $0.68 \pm 0.05$  g (female canary),  $0.53 \pm 0.04$  g (male zebra finch), and  $0.51 \pm 0.04$  g (female zebra finch).
16. In the case of the motor nucleus of the hypoglossus (nXII), the volume of the entire nucleus was measured in the canaries. It includes an anterior portion innervating the tongue, and a caudal portion (tracheosyringeal portion, nXIIts), which is composed of the motoneurons innervating the vocal organ (syrinx). In the zebra finches, only the volume of nXIIts was measured. This was possible because in each of five males and females, the tracheosyringeal branch of the hypoglossus had been cut unilaterally (three male-female pairs on the left, two on the right) 7 to 9 days before the birds were killed. Under this procedure, the ipsilateral syringeal motoneurons of nXIIts become chromatolytic and swell and serve as a guideline for the limits of nXIIts on the nonchromatolytic side. Since the swelling also enlarges the volume of nXIIts, the values presented in figure 25.2 are derived from twice the volume of the nonchromatolytic side of nXIIts for each zebra finch. In both species the perimeter drawn around the motor nucleus circumscribed all of the motoneuron cell bodies but not the neuropil that surrounds the motor nucleus.
17. The motor nucleus nXIIts innervates the syrinx in both canaries and zebra finches (4, 19).
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19. In the pigeon, Rt is part of the tectofugal visual pathway [H. J. Karten and A. M. Revzin, *Brain Res.* 2, 368 (1966); A. M. Revzin and H. J. Karten, *ibid.* 3, 264 (1966–1967)], and SpM receives input from the telencephalon and projects to the cerebellum [H. J. Karten and T. E. Finger, *ibid.* 102, 335 (1976)].
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21. In females, the mean volume of nXII on the right was  $0.0332 \pm 0.0021$  mm<sup>3</sup> (mean  $\pm$  standard deviation), compared with  $0.0436 \pm 0.0053$  mm<sup>3</sup> on the left (two-tailed *t*-test,  $P < .001$ ). In males, the volume was  $0.0495 \pm 0.0074$  mm<sup>3</sup> on the right and  $0.0526 \pm 0.0075$  mm<sup>3</sup> on the left ( $P < .02$ ). Since neurons on one side of nXIIts were swollen and chromatolytic in the zebra finches (16), we could not assess any possible differences in volume between the sides of the brain in that species.
22. F. Nottebohm and M. Nottebohm, *J. Comp. Physiol.* 108, 171 (1976).
23. The syringeal musculature is larger in male canaries and zebra finches than in females; we have not calculated the male/female ratio of syringeal muscle volume.
24. Observations (4) are of male canaries. As in males, area X of female canaries also receives a discrete fiber projection from the ipsilateral HVc (F. Nottebohm, unpublished observations).
25. F. Nottebohm, in preparation; A. P. Arnold, in preparation.
26. In figure 25.2,  $N = 5$  for measurements on canaries, and  $N = 4$  to 7 for zebra finches.
27. We thank C. M. Leonard and P. Marler for discussion of our results and Y. Holland for technical assistance. Supported by NIMH grant 18343 to F.N. A.P.A. was a NIMH postdoctoral fellow (No. 00559).



## Introduction

The idea of critical periods for sexual differentiation in mammals is a widely accepted one. Typically, the levels of gonadal steroids during a perinatal period are thought to set the CNS on a masculine or feminine pattern of differentiation (5, 22). According to this classical view, the morphological characteristics of pathways serving typically male or female functions are not further affected in adulthood. Data presented here indicate that in one species of bird gonadal hormones can induce gross morphological changes in the adult brain.

Two vocal control nuclei of the canary telencephalon, hyperstriatum ventrale, pars caudale (HVc) and nucleus robustus archistriatalis (RA) (16), are larger in males, that learn complex songs, than in females, that normally do not sing (14). However, adult female canaries can be induced to sing by testosterone treatment (4, 8, 12, 19). It is shown here that testosterone levels adequate to induce song in adult female canaries will also induce in them marked growth of vocal control nuclei HVc and RA. The magnitude of this effect is comparable, but of reversed sign, to that following early castration in males.

## Methods

### Animals and Hormone Treatment

All birds used were of the Belgian Wasmuschlag strain. The original stock was imported from Belgium in 1967. Those birds and their descendants have been kept at Rockefeller University as a close bred colony since that time. In a first, pilot, experiment 6 intact females received when 2 years old a 10 mg pellet of testosterone propionate (Oreton, Schering Corp.); 4–5 weeks later these birds were sacrificed. No blood androgen levels were obtained. The brain and behavior of these 6 birds was compared with that of 5 intact females of the same age.

The remainder of the experiments was conducted with 1-year-old birds, of which 16 were females and

20 males. Six of the females were kept intact while the other 10 had their single ovary removed 6–18 days after hatching. At 11 months of age each of the ovariectomized females received a silastic capsule (Storz Instrument Co., St. Louis, Mo.; i.d., 0.030 in., o.d. 0.065 in.) containing cholesterol ( $n = 5$ ) or testosterone ( $n = 5$ ). These capsules were implanted subdermally, overlying the pectoral muscle mass. Capsules were made according to Legan et al. (11) and contained 5 mm of packed steroid. In preliminary experiments constant levels of circulating testosterone were maintained by the silastics for at least one month. Observations were also conducted on 10 sibling pairs of males. One bird in each pair was kept intact, the other one had both testes removed 5–10 days after hatching. All birds in this second group of experiments were sacrificed at 12 months of age.

### Treatment of Tissues

Birds were sacrificed by an overdose of anesthesia (ether). Before the birds expired, and as part of another study, a sample of blood was removed by intracardiac puncture of the right auricle. Dr. Cheryl Harding used this blood to determine blood androgen levels via the radioimmunoassay technique. Details on this technique are given elsewhere (13). Birds were then perfused with saline through the left ventricle, followed by perfusion with 10% formalin in physiological saline. The brains were removed, blocked, fixed, embedded in gelatin albumen and sectioned transversely as described in Stokes et al. (20). The sections were mounted on glass slides. All brains were weighed before being embedded. Serial sections were cut with a repeat thickness of 50, 50 and 25  $\mu\text{m}$ ; only one of the 50  $\mu\text{m}$  series was mounted, stained with cresyl violet for cell bodies, and viewed under the light microscope. The distance between the surface of two successive stained sections was 125  $\mu\text{m}$ , and this figure was used in the volume reconstructions described next.

Both HVc and RA have discrete boundaries. HVc is limited dorsally by the floor of the telencephalic ventricle, and ventrally by a lamina of fibers and small

cells. RA is surrounded almost completely by a capsule of fibers and small cells (16). The volume of HVc, RA and of two cytoarchitectonically discreet midbrain nuclei not involved in vocal control, rotundus (Rt) and spiriformis medialis (SpM) (9, 10, 18), was measured as follows. A microprojector (Bausch and Lomb) projected magnified ( $\times 57$ ) images of cresyl violet stained sections on drawing paper. A perimeter was then drawn around the regions of interest, and the area enclosed was measured with a polar planimeter. These areas were then multiplied times  $125 \mu\text{m}$ . The sum of all such products for a given brain region was an estimate of its volume. Such estimates were obtained for the same structure on the right and on the left half of the brain. Total volume estimates correspond to the additions of right plus left volumes.

### Sound Recording and Analysis

Canary song develops gradually during ontogeny. "Subsong" refers to the earliest, low amplitude and highly variable stage of song development. It leads into "plastic song" which is louder, more structured and less variable. Six to eight months after hatching plastic song leads to the stereotyped song of adult males. The vocal behavior of all birds included in the present study was monitored. Birds that sang had 600 sec of song recorded on tape at a speed of 3.5 cm/sec. Their adult song was scored by counting the number of different syllable types, as described in Nottebohm and Nottebohm (15).

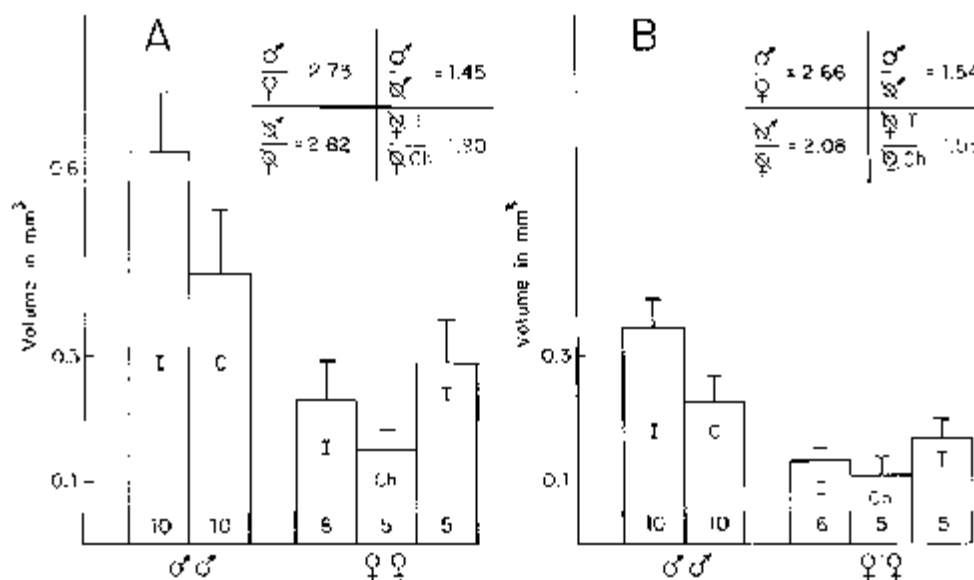
## Results

### Brain Weights

Brains were weighed after being fixed in sucrose-formalin. Means and standard deviations for the brain weights of males and females in the different treatment groups were as follows: intact males, 0.68 g (S.D. = 0.03); castrate males, 0.66 g (0.07); intact females, 0.80 g (0.06); cholesterol treated ovariectomized females, 0.76 g (0.05); testosterone treated ovariectomized females, 0.73 g (0.09). There was no statistical difference between groups of a same sex (two-tailed *t*-test,  $P > 0.10$ ). However, male brains as a group ( $n = 20$ ; mean = 0.67 g, S.D. = 0.06) weighed less than those of females as a group ( $n = 16$ ; mean = 0.76 g, S.D. = 0.07), and this difference is statistically significant ( $t = 4.3$ ,  $P < 0.001$ ). This difference may have resulted from the fact that the assistant who removed the brains from the skull improved with experience in his ability to include longer and longer segments of spinal cord! The male brains were as follows: intact males, 0.68 g (S.D. = 0.03); castrate males, 0.66 g (0.07); intact reported non-significant brain weight differences of a smaller magnitude, and on that occasion the males had the heavier brains (14).

### HVc and RA Volume

The HVc and RA volumes of the castrated males were in all cases smaller than those of their intact siblings (figure 26.1). The difference in volume between the



**Figure 26.1**

A: mean total (right + left) adult volumes of HVc. The vertical line above the bar is the standard deviation of the individual values about the mean. B: RA volumes. Abbreviations: I, intact males (♂) or females (♀); C, males castrated 5–10 days after hatching (♂). Ch and T, females ovariectomized (♀) 6–18 days after hatching, treated with cholesterol or testosterone at 11 months (see text). The number of individuals in each group is shown at the bottom of each histogram column. All ratios were obtained between means shown in the respective histograms. For the ♂/♀ ratio the ♀ symbol refers to the ovariectomized females treated with cholesterol.

two groups was highly significant (Wilcoxon matched-pairs signed-ranks test,  $P < 0.01$ ). The corresponding ratios for Rt and SpM were 0.98 and 0.96,  $P > 0.05$ .

Of the two groups of early ovariectomized females, HVc and RA were 90% and 53% respectively larger in the testosterone- than in the cholesterol-treated birds, as shown in figures 26.1 and 26.2 (for HVc,  $t = 4.17$ ,  $P < 0.02$ ; for RA,  $t = 3.04$ ,  $P < 0.02$ ). Comparable ratios (testosterone-treated/cholesterol-treated) for Rt and SpM were 0.95 and 1.06 respectively ( $P > 0.20$ ). The volumes of HVc and RA of the intact 1-year-old untreated females fell between those of the cholesterol- and testosterone-treated ovariectomized females. Comparing the volumes of HVc and RA of the intact 1-year-old untreated females with those of the two ovariectomized groups, yielded only one significant difference: HVc was found to be 50% larger in intact untreated females than in ovariectomized females treated with cholesterol ( $t = 2.53$ ;  $P < 0.05$ ).

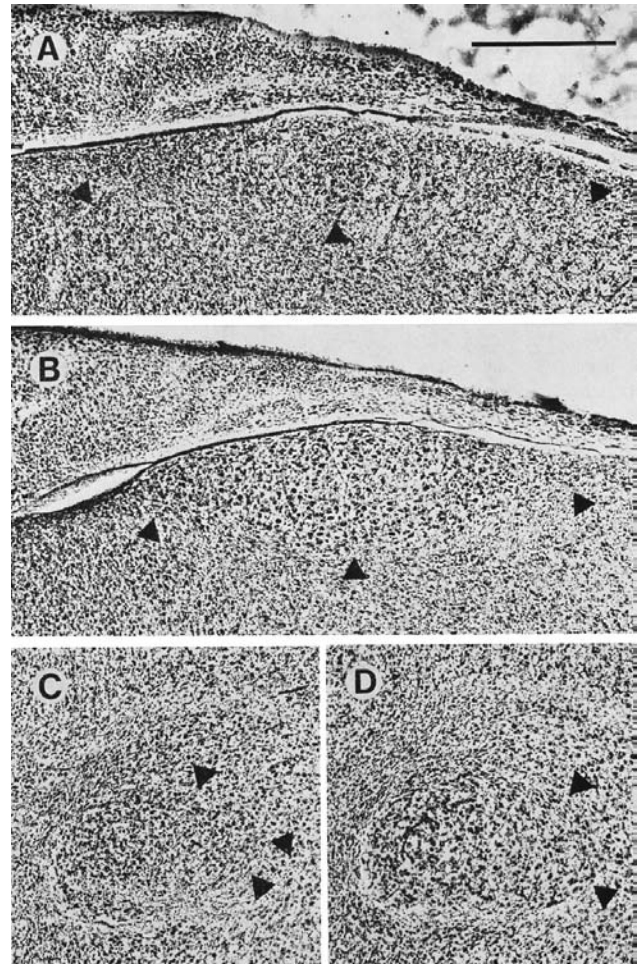
Testosterone also had an effect on the HVc and RA volumes of the 2-year-old intact females, where these two structures were 50% and 69% larger, respectively, than in the untreated 2-year-old intact females, ( $P < 0.02$  and  $P < 0.01$  respectively,  $t$ -test).

#### Blood Androgen Levels

Blood androgen levels were obtained only for the 36 1-year-old birds. Testosterone (T) levels are given first, in ng/ml serum, followed by dihydrotestosterone (DHT) levels; each figure is followed by S.E. of the mean: intact males,  $3.2 \pm 0.57$  and  $0.7 \pm 0.13$ ; castrate males,  $0.09 \pm 0.09$  and  $0.13 \pm 0.10$ ; intact females  $0.01 \pm 0.01$  and  $0.00 \pm 0.00$ ; cholesterol-treated ovariectomized females  $0.07 \pm 0.05$  and  $0.23 \pm 0.1$ ; testosterone-treated ovariectomized females,  $2.33 \pm 0.39$  and  $0.59 \pm 0.23$ . It will be noted that testosterone treatment of ovariectomized females brought blood T and DHT levels well within the physiological range of intact males.

#### Song

The 10 intact males had song repertoires of 19–41 different syllable types, with a mean of 26 (S.D. = 7.1). The castrated siblings went through the subsong and plastic song stages of song development. However, at the time the intact controls were singing profusely the stereotyped patterns typical of a bird in full reproductive condition, the castrates were silent, and failed to develop stable adult song before they were sacrificed. Of the female birds only the two groups treated with testosterone produced male-like song. The birds started to sing about a week after receiving the testosterone implant and remained in song until sacrificed. Their song repertoires were abnormally simple. The 1-year-old ovariectomized females that received testosterone



**Figure 26.2**

A–D: cresyl violet-stained, 50  $\mu$ m-thick sections from 4 different female canaries ovariectomized 7–9 days after hatching and sacrificed at 12 months of age. A and B: cross-sections of HVc. C and D: cross-sections of RA. Birds A and C received a subcutaneous silastic implant of cholesterol at 11 months. Birds B and D received a subcutaneous silastic implant of testosterone at 11 months. The anterior–posterior level of sectioning corresponds to that showing the maximal cross-sectional area of HVc or RA for each of these birds. Birds were chosen to represent median HVc and RA volumes for the cholesterol- and testosterone-treated groups. Notice that the distance between cell somas of HVc and RA is greater in testosterone than in cholesterol-treated females. The horizontal calibration bar corresponds to 0.5 mm. In A and B black triangles point to ventral boundary of HVc. In C and D black triangles point to dorsolateral boundary of RA. The remainder of the RA boundary can be recognized as a pale fibrous lamina.

had syllable counts ranging from 4 to 6 different syllable types per bird, with a mean of 4.6, S.D. = 0.89. The 2-year-old intact females treated with testosterone had syllable repertoires that ranged from 4 to 11 different syllable types, with a mean of 7.2 (S.D. = 2.6). The syllable repertoire of both groups of females was significantly smaller than that of the intact males ( $t = 7.09$  and  $6.7$ , respectively;  $P < 0.001$ ). At the time the testosterone-treated females were sacrificed, syllable structures still remained unstable. The cholesterol-treated females did not sing.

## Discussion

Earlier experiments had already shown that female canaries can be induced to sing by testosterone treatment (4, 8, 12, 19). It also was known that song induced in this manner was simpler than that of intact adult males (4). What comes as a surprise is that the same physiological levels of androgen which induce song in ovariectomized females also trigger a dramatic increase in the size of two telencephalic nuclei, HVC and RA. Both the anatomical and behavioral effects also occur in testosterone-treated 2-year-old intact females. The magnitude of volume increases induced by testosterone is comparable, but of reversed sign, to that which follows early castration in males.

To my knowledge this is the first time that a steroid hormone is shown to induce in adulthood changes in brain anatomy of a magnitude usually associated with much earlier stages of sexual differentiation (1, 6, 17, 21).

The effects of the exogenous testosterone on female RA and HVC volumes could have been mediated by its metabolites,  $5\alpha$ -dihydrotestosterone or estradiol. Estradiol seems to be an unlikely candidate in this case since this hormone can be expected to abound in female canaries in reproductive conditions, yet such birds do not sing.

There are other grounds to suspect that the changes observed in HVC and RA result from genomic effects of androgen. The nuclei of cells in HVC and RA of adult male zebra finches concentrate label after systemic injections of tritiated testosterone, but there is little or no accumulation of label after injections of tritiated estradiol (2, 3). This pattern of selective uptake has also been observed in canaries (Kelley and Nottebohm, in preparation).

Though normal adult volumes of HVC and RA in males and adult volume of HVC in females require the presence of intact gonads, the *ratio* of male to female volumes of these structures is little affected by early post-hatching gonadectomy (figure 26.1). Presumably still earlier events have already biased development of

these structures differently in the two sexes, as shown by Gurney and Konishi in the zebra finch (7).

The hormone-induced differences in volume of HVC and RA described here may reflect changes in numbers of neurons or glia, or changes in size and number of neuronal or glial processes. Studies are underway to explore these various possibilities. One effect of testosterone treatment readily observed in figure 26.2 is the increased distance between cell somas of HVC and RA. Histological treatment received by material used in this study did not permit an accurate quantification of this effect.

Finally, a caveat. Under the conditions of the experiments described here testosterone induced adult song and the growth of HVC and RA. We do not know which of these two effects occurred first, or whether they were simultaneous. The quest for understanding the causal links that relate testosterone to song and to changes in brain structure has just begun.

## Acknowledgements

I am very thankful to Susan Marcinkowski and Constantine Pandazis for the skill and dedication they brought to all aspects of histology, measurement of results and photography. Timothy DeVoogd, M. E. Seeber de Nottebohm and Dominique Toran-Allerand provided helpful comments on the manuscript. This work was supported by PHS Grants 5R01 MH18343 to F.N. and 5S07 RR07065 to Rockefeller University, and by Rockefeller Foundation Grant RF70095 for Research in Reproductive Biology.

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Successful reproduction in sexual animals depends on mating with members of the same species. In many species, females are able to recognize conspecific males on the basis of their species-specific courtship signals. Numerous studies have documented the selectivity of neurons in sensory brain regions for conspecific signal features (1). The role played by brain nuclei in the behavioral recognition of conspecific mating signals by females, however, has received scant attention.

Song production in male songbirds is controlled by a network of brain nuclei (2). In many species females possess the same network of song nuclei as conspecific males, albeit they are of smaller size (3). The presence of these nuclei in the brains of females that do not normally sing is puzzling. Why should these females possess such song nuclei at all? No function for these song nuclei in females has yet been demonstrated empirically.

One possibility is that these song nuclei play a role in song perception in females (4). In males of several songbird species, neurons in several of these nuclei receive and respond to input from the auditory system (5). Neurons in male song nuclei respond more selectively to conspecific song stimuli than do neurons in purely auditory nuclei. For example, the caudal nucleus of the ventral hyperstriatum (HVC) of males is critical for song production. The HVC receives auditory input from field L, the primary forebrain auditory nucleus. Multineuron clusters in HVC respond preferentially to an individual bird's own song (6). Neurons in field L do not respond selectively to an individual's song, but show more generalized response properties (6).

Previous studies have not shown whether HVC or other song nuclei play a role in conspecific song perception in female birds, as they do in males. To examine this hypothesis, I tested the behavioral responses of female roller canaries (*Serinus canarius*) to playback of conspecific and heterospecific songs, before and after selective lesions of HVC. I found that lesions of part of HVC eliminated the discrimination between these signals without disrupting behavioral responses to song. The results indicate that HVC does act in song

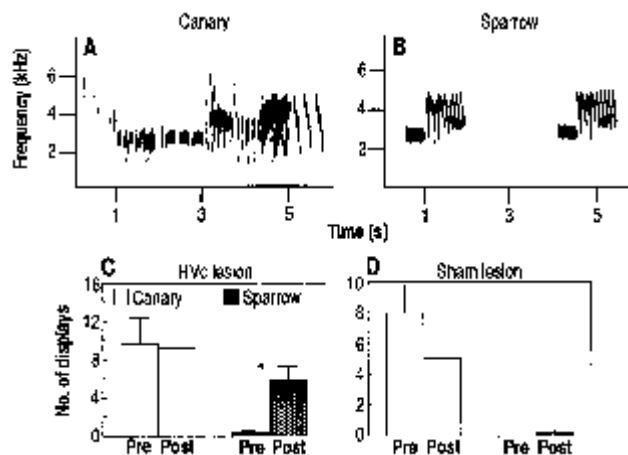
perception in female birds that do not normally sing (7).

Adult female canaries ( $n = 17$ ) were brought into breeding condition with silastic implants of estradiol placed over the pectoral muscle (8). Each bird was housed individually in a sound-isolated chamber equipped with a loudspeaker. Starting 1 week after hormone implant, conspecific (roller canary) and heterospecific (white-crowned sparrow, *Zonotrichia leucophrys*) songs were played to the females (figure 27.1, A and B) (9). As a behavioral measure of song perception, I scored the number of copulation solicitation displays performed by the focal female during a 5-min test period. In this stereotyped display, performed in nature just before copulation, a female responds to conspecific song by thrusting her breast forward, raising her tail sharply, and holding her wings out to the side while vibrating them rapidly. This display has been used to test female responses to song in several species (10).

Playback tests were repeated every 2 days until the first day that a female canary indicated sexual receptivity by giving at least five solicitation displays during the 5-min presentation of canary song. When she attained this response level, she was anesthetized with Equithesin and received bilateral electrolytic lesions directed at the forebrain vocal nucleus HVC ( $n = 13$  birds) (11). Starting 2 days after the lesion was made, she was again tested for responses to canary and white-crowned sparrow song. Testing continued at 3-day intervals for at least two more weeks. The remaining four birds received sham lesions (12). The observer was blind as to whether the subject was lesioned or sham-operated.

Before lesion of the HVC, female canaries discriminated between conspecific and heterospecific song. No female gave more than one solicitation display to white-crowned sparrow song per 5-min test period; this always occurred within the first 10 s of the onset of the playback. Playback of male canary song, however, evoked strong responses (that is,  $\geq 5$  displays/5 min) from female canaries (figure 27.1, C and D) (13).

Histological analysis of brains indicated that in five females the lesions eliminated the medial and caudal



**Figure 27.1**

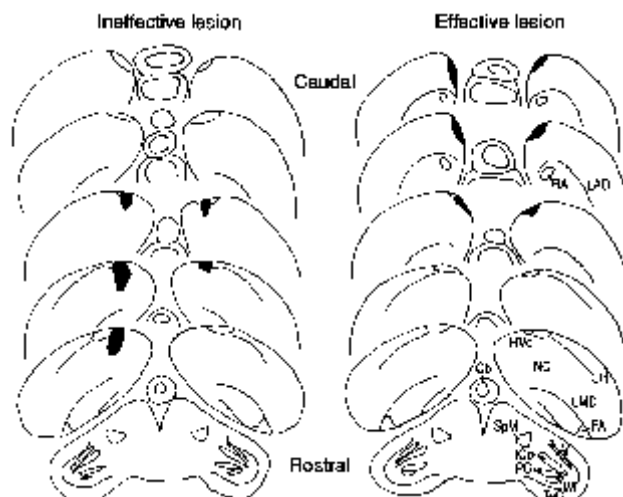
Sonograms of (A) canary song (segment) and (B) white-crowned sparrow song used as playback stimuli. (C) Median number of copulation solicitation displays (group  $\pm 1$  SEM) given by five female canaries to playback of canary and sparrow songs pre- and post-lesion of HVC. (\* $P < 0.02$ , paired  $t$  test on median number of displays.) (D) Displays by four female canaries to canary and sparrow songs before and after sham lesions.

portions of HVC on both sides (figure 27.2). These birds altered their behavior dramatically; they responded strongly to white-crowned sparrow song (figure 27.1C; pre- versus post-lesion responses to sparrow song,  $P < 0.02$ , paired  $t$  test, two-tailed). After lesions, the number of solicitation displays to canary song and to sparrow song did not differ ( $P > 0.05$ ). Responses to canary song before and after lesions showed no statistically significant difference ( $P > 0.05$ ) (14).

In the remaining eight lesioned birds, the medial and caudal portions of HVC remained intact on one or both sides (figure 27.2). These females continued to respond only to canary song, even if the lateral and rostral portions of HVC were lesioned bilaterally.

Birds that received sham lesions also continued to respond strongly only to conspecific song (figure 27.1D; post-lesion responses to canary song versus white-crowned sparrow song,  $P < .001$ ).

Three of the five birds that altered their behavior after HVC lesions were observed over relatively long time periods post-lesion (20 days, 51 days, and about 180 days). They continued to respond to sparrow song throughout these periods. None of the sham-lesioned or ineffectively lesioned birds ever altered their responses to heterospecific song. Comparison of these latter two groups with the former group thus indicates that lesions of HVC had a pronounced and fundamental effect on species-specific song responses (15). The prolonged duration of altered response in effectively lesioned birds shows that the change in song discrimination was not due to transient, nonspecific effects such as post-surgical trauma.



**Figure 27.2**

Serial reconstructions of typical lesions that were ineffective or effective at evoking solicitation displays to white-crowned sparrow song by female canaries. Black indicates site of lesion. Cb, cerebellum; FA, fronto-archistriatal tract; HVC, caudal nucleus of the ventral hyperstriatum; ICo, intercollicular nucleus; IM, magnocellular nucleus of the isthmus; IPC, parvocellular nucleus of the isthmus; LAD, dorsal archistriatal lamina; LH, hyperstriatal lamina; LMD, dorsal medullary lamina; NC, caudal neostriatum; RA, robust nucleus of the archistriatum; SpM, medial spiriform nucleus; and TeO, optic tectum. Scale bar, 1 mm.

HVC lesions may have eliminated the ability of female canaries to perceive differences between conspecific and heterospecific songs. Alternatively, these lesions may simply have lowered the threshold for responding to acoustic stimuli in general. The latter explanation is unlikely, however, because lesions of HVC only increased the strength of response to heterospecific song. The results are more consistent with the conclusion that lesions disrupted the role of HVC in the perception of conspecific song in female canaries (16).

The response measure, copulation solicitation behavior, did not distinguish between two components of the perceptual process. Did effectively lesioned females actually fail to discriminate species differences in song, or did they just fail to respond to those differences? Psychophysical tests of song perception in HVC-lesioned birds can address these questions.

It will be interesting to examine the physiological response selectivity of single-units and multi-unit clusters in female HVC. I predict that female HVC neurons will respond to a broader array of conspecific song stimuli than is observed in male HVC, in which neurons respond specifically to the bird's own song.

A challenge remaining is to determine how song perception develops in female birds that do not normally sing. It has been proposed (17) that neurons in male HVC acquire their pronounced song selectivity through a mechanism similar to that presented in the motor

theory of human speech perception (18). This theory posits that, to perceive a song syllable, a bird must first convert the sound it hears into the motor commands required to produce that sound. Male HVC neurons develop song selectivity during the sensorimotor phase of song learning (6). Song production and song perception are thus viewed as being functionally linked in males. Female birds that do not normally sing presumably do not develop the entire set of motor commands required in male HVC for the production of full adult song. However, females learn to perceive even subtle differences among the songs of conspecific males (8, 19). It thus seems unlikely that such perceptual learning in female birds can be explained fully by a motor theory of song perception, at least as proposed for males (17). Rather, the sexes may differ in the mechanisms by which neurons in song nuclei develop their selectivity to conspecific courtship signals.

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7. Captive female canaries may produce a song-like vocalization if isolated for several months. This vocalization is much simpler and less stable in structure, and is produced less often, than normal male song [A. Pesch and H.-R. Guttinger, *J. Ornithol.* 126, 108 (1985); Nottebohm (2); personal observation]. There are no reports of song in wild female canaries.
8. All birds were kept in a short-day photoperiod (light:dark, 8:16 hours) for at least 2 months before the start of the experiment. They were then gradually shifted to a long-day photoperiod (light:dark, 14:10 hours) to bring them into breeding condition. When a female developed a vascularized brood patch, she received two subdermal implants of estradiol (inner diameter, 0.76 mm; outer diameter, 1.65 mm; length = 12 mm) to induce copulation solicitation behavior in response to conspecific song.
9. The conspecific stimulus was a 5-min sequence of male roller canary song containing 19 syllable types and consisting of bouts of  $4.8 \pm 2.3$  phrases ( $x \pm SD$ ), lasting  $5.4 \pm 3.0$  s, and separated by silent intervals of  $1.8 \pm 0.9$  s. [See F. Nottebohm and M. E. Nottebohm, *Z. Tierpsychol.* 46, 298 (1978)]. In preliminary studies, female canaries only responded to songs produced by males of the same strain. The heterospecific stimulus was a white-crowned sparrow song recorded from the *Z.l. nuttalli* dialect in northern California. [See L. Baptista, *Univ. Calif. Publ. Zool.* 105 (1975)]. White-crowned sparrow song was used because it contains frequency-modulated syllables as does canary song but has a very different syntactical structure. The single sparrow song lasted 1.55 s, repeated at intervals of 2.45 s for 5 min. Songs of both species were played at a maximum root-mean-square amplitude of 90 dB sound pressure level at 50 cm. The two songs were presented in random order each day, at 3-hour intervals to minimize facilitation or habituation effects.
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11. Lesions were made by passing 100  $\mu$ amps of anodal direct current for 2 min through insulated tungsten wire. Sites of lesions were confirmed histologically.
12. For sham lesions, birds were anesthetized, and an electrode was lowered to the stereotactic coordinates of HVC, but no current was delivered to the electrode.
13. For statistical analysis I used the median number of each bird's displays obtained from her full set of pre-lesion and post-lesion playback sessions. There were 1 to 4 pre-lesion and 1 to 13 post-lesion playback sessions per bird.
14. Ranges for the median number of solicitation displays are as follows: (i) effectively lesioned birds, pre-lesion canary song, 5 to 20 displays; post-lesion canary song, 4 to 16.5 displays; pre-lesion sparrow song, 0 to 1 display; post-lesion sparrow song, 3 to 11 displays; (ii) sham-lesioned birds, pre-lesion canary song, 5 to 12 displays; post-lesion canary song, 5 displays; pre-lesion sparrow song, 0 display; post-lesion sparrow song, 0 to 1 display.
15. It is unlikely that lesions acted by disrupting fibers of passage between song nuclei other than HVC. Axons projecting from lateral MAN (magnocellular nucleus of the anterior neostriatum) to RA travel lateral and ventral to HVC [F. Nottebohm, D. Kelley, J. Paton, *J. Comp. Neurol.* 207, 344 (1982)]. Only lesions of medial caudal HVC induced responses to sparrow song. Projections between other song nuclei do not pass adjacent to HVC.
16. To determine whether effective lesions of HVC eliminated all song discrimination, I played the songs of different bird species to one female canary 7 months post-lesion. She responded strongly ( $\geq 5$  solicitation displays during 5 min of playback) to songs of the northern mockingbird (*Mimus polyglottos*), the bay wren (*Thryothorus nigricapillus*), and the buff-breasted wren (*T. leucotis*). These songs have different syntactical structures, but are similar to each other and to canary song in that each has syllables with pronounced frequency modulation. The same female did not respond to rufous-and-white wren (*T. rufalbus*) song, which contains little frequency modulation. These observations suggest that lesions of medial caudal HVC eliminated the species-specificity of responses and resulted in a more generalized pattern of signal discrimination. The altered pattern of response may be related functionally to reports that HVC neurons respond more selectively to species-specific song stimuli than do neurons in the afferent auditory nucleus, field L (6).
17. H. Williams and F. Nottebohm, *Science* 229, 279 (1985).
18. A. M. Liberman, *Am. Psychol.* 37, 148 (1982).
19. D. Miller, *Anim. Behav.* 27, 376 (1979); *Nature* 280, 389 (1979); see M. Baker et al. (10).
20. Thanks to M. Beecher, S. Bottjer, K. Canady, G. Klump, P. Loesche, K. Nordeen, G. Rose, E. Rubel, D. Sengelaub, S. Volman, H. Zakon, and two anonymous referees for comments. S. Bottjer and A. Arnold offered advice during preliminary studies. B. Nalls provided technical assistance. Supported by NIH DC 00487, NSF BNS 86-02469, and a Sloan fellowship.



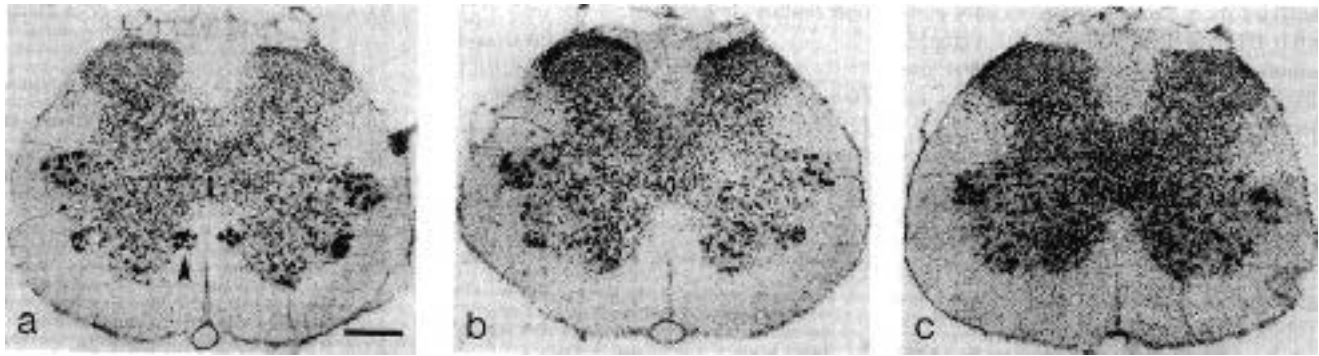
Because of the obvious sex differences in the behavior of vertebrates, one might expect to find differences in the central nervous system (CNS) of the two sexes. Raisman and Field (1) reported that in rats the “striatal portion” of the preoptic area (POA) has more synapses from nonamygdaloid sources in females than in males. Other anatomical sex differences described in the nervous system include the number of autonomic preganglionic neurons in cats, and the dendritic field patterns in the POA of hamsters (2). Nottebohm and Arnold (3) reported gross sexual dimorphism in the size of certain brain nuclei of song birds. These nuclei are larger in males and are known to play a role in the singing behavior which only males display. Gorski et al. (4) found a strikingly dimorphic nucleus in the rat POA which is larger in males than in females. Because the last two dimorphisms are easily detected, they provide convenient measures of the process of sexual differentiation of the brain, and therefore study of these systems may lead to a better understanding of the factors critical to sexual differentiation of vertebrate behavior. We now report a prominent dimorphism in a motor nucleus of the rat spinal cord, the neurons of which accumulate radioactivity after injections of tritiated androgens but not estradiol. This dimorphism is useful for studying sexual differentiation of the CNS because it offers advantages distinctive to motoneurons, for example, readily determined behavioral function, electrophysiological accessibility due to the large somas, relatively simple inputs and outputs, and ease of study in early development.

Motoneurons were identified by injecting horseradish peroxidase (HRP) into each of the three striated muscles attached to the rat penis: the ischiocavernosus (IC), bulbocavernosus (BC), and levator ani (LA) (5). The muscles of 30 male anesthetized Sprague-Dawley rats were injected with 3 to 50  $\mu$ l of a 30 percent solution of HRP (Sigma type VI) in saline by means of a 10- $\mu$ l syringe under a dissecting microscope (6). Multiple injections were used to distribute the HRP evenly throughout the muscle, and afterward the exposed perineal region was flushed with saline (7). The animals were killed 24 hours later and the retrogradely trans-

ported HRP was stained in the spinal cord segments caudal to thoracic 13 (8).

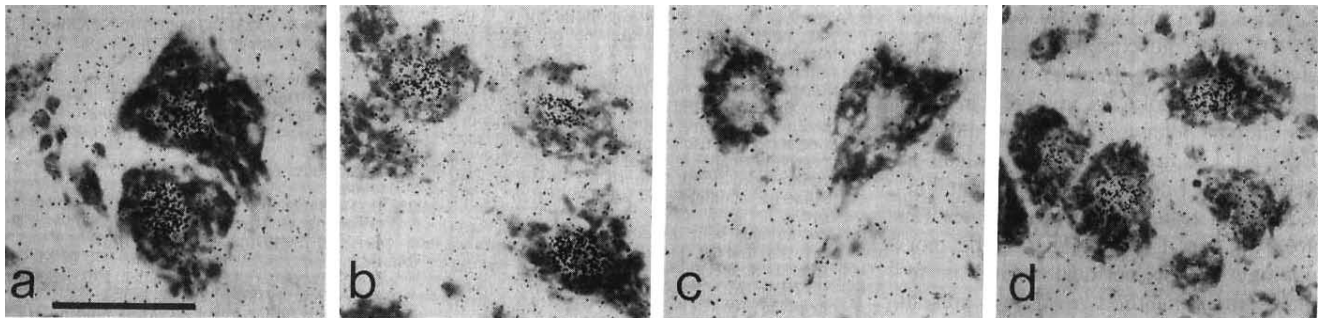
After the injection of HRP into IC, retrogradely labeled motoneurons were found in the extreme ventrolateral quadrant of the ventral horn in the fifth and sixth lumbar spinal segments ( $L_5$  and  $L_6$ ). Injection of HRP into either the BC or LA resulted in labeled cells being found in the dorsomedial portion of the ventral horn, 50 to 250  $\mu$ m from the midline and 200 to 400  $\mu$ m below the ventral margin of the central canal (figure 28.1a, arrow). These latter neurons form a compact nucleus extending 1.5 mm from caudal  $L_5$  to rostral  $L_6$ , on the border of the ventral funiculus of white matter. The neurons of this nucleus are large (30 to 50  $\mu$ m in diameter), multipolar, and stain densely for Nissl substance. Because we believe this nucleus is hitherto undescribed, we propose naming it the spinal nucleus of the bulbocavernosus (SNB). This name seems appropriate since other evidence suggests that the LA may more properly be called the dorsal bulbocavernosus (9). Comparison of thionin-stained  $L_5$  and  $L_6$  cord sections from male and female rats revealed that in females there are fewer cells in the region (10) and the few cells present are smaller than those of the male SNB, resulting in the apparent absence of the SNB in female rats (figure 28.1, a and b).

Because some motoneurons of the male rat spinal cord accumulate radioactivity after injection of tritiated dihydrotestosterone (DHT) (11), and because the position of cells in the SNB make them readily distinguishable, we used autoradiography to determine whether these particular motoneurons accumulate hormones. Adult male rats were castrated and adrenalectomized, then maintained on drinking water containing 0.9 percent saline. Two days later an intra-atrial catheter was implanted, and 24 hours after catheter implantation the animals were injected with tritiated DHT, testosterone (T), or estradiol (E) (1.2 nmole per 100 g of body weight in a 0.3-ml vehicle of 50 percent ethanol) (12). One hour after injection the rats were decapitated and the lumbar and sacral sections of the spinal cord were removed. Each cord was cut into blocks two spinal segments long and frozen



**Figure 28.1**

Sections from the lumbar segments of the rat spinal cord. (a) and (b) Thionin-stained 50- $\mu$ m transverse sections from male and female fifth lumbar segments, respectively. The arrow in (a) points to the left spinal nucleus of the bulbocavernosus (SNB) of the male. Note the virtual absence of the SNB in the female cord in (b). Scale bar, 400  $\mu$ m. (c) Section of the lumbar spinal cord of a genetically male rat which because of the testicular feminization (*Tfm*) mutation, possesses few androgen receptors. Despite the fact that this is a genetic male, the SNB is absent, implying that the interaction of androgens with their receptors is important to the development of the SNB. Normal male littermates of the *Tfm* males have a normal SNB. Magnification as in (a) and (b).



**Figure 28.2**

Autoradiograms from the fifth and sixth lumbar segments of the male rat spinal cord, stained with thionin (scale bar, 50  $\mu$ m). (a to c) Cells of the spinal nucleus of the bulbocavernosus (SNB) after injections of tritiated DHT, T, and E, respectively. Note accumulation of hormone over the relatively unstained nucleus in (a) and (b), but not in (c). (d) Ventrolateral motoneurons of the fifth lumbar segment after injection of tritiated DHT. Exposure periods: (a) 211, (b) 67, (c) 64, and (d) 211 days.

with dry ice. The tissue was cut in 6- $\mu$ m transverse sections in a cryostat at  $-20^{\circ}\text{C}$ , and placed on microscope slides previously coated with nuclear track emulsion. The autoradiograms were photographically developed 29 to 258 days later, counterstained with thionin, and examined under a microscope (13).

To decide whether a given cell was significantly labeled, we used a Poisson model of the distribution of reduced silver grains. The expected number of grains over the nucleus of a cell was calculated from the density of grains over the background (adjacent unstained neuropil) and the area of the cell's nucleus. This expected number was then used as the mean of a Poisson distribution describing the number of grains that would occur over that cell's nucleus by chance. If the actual number of silver grains over the nucleus was more than would be expected by chance for that Poisson distribution ( $P < .01$ ), then the cell was considered labeled. This criterion is less stringent than the more frequently used criterion of five times background den-

sity (14), but the Poisson distribution provides a more accurate evaluation of whether a cell is labeled. Each hormone was injected into three males and at least 50 SNB cells were analyzed from each. The SNB neurons were easily identified by location, large somas, multipolar shape, and dense staining. For purposes of comparison, the nuclei of 50 large, multipolar, densely staining motoneurons in the ventrolateral portion of the L<sub>5</sub> and L<sub>6</sub> cord sections were examined. This population includes but is not exclusively the motoneurons of IC. The observer analyzing the autoradiograms was unaware of which hormone had been injected (figure 28.2).

The percentages of SNB cells labeled by the three hormones are shown in table 28.1, where both the Poisson and the five-times-background criteria are listed. With either criterion, more SNB cells are labeled after DHT or T injections than E (15). Injection of E resulted in densely labeled cells being found in the dorsal horn and lamina X (16). The SNB neurons

**Table 28.1**

Percentage of SNB or VLMN cells that are labeled after injection of tritiated hormone, according to either the Poisson or five-times-background criteria.

Hormone	SNB		VLMN	
	Poisson	5×	Poisson	5×
DHT	97.6	68.6	96.4	34.9
T	96.0	40.1	77.4	7.7
E	3.3	0	5.8	0

accumulated hormone more heavily after DHT than T injections, since a greater proportion of SNB cells reached the more stringent five-times-background criterion after DHT injection (15). More of the SNB cells accumulated T or its metabolites than did the ventrolateral motoneurons (VLMN) (table 28.1). The SNB cells also accumulated radioactivity more densely than VLMN cells after DHT or T injections since a greater number of the SNB cells reached the five-times-background criterion after injection of these hormones (15).

Because the SNB cells of adult rats accumulate androgens but not estrogens, one might expect that androgens play a role in the sexually dimorphic development of the SNB. Thus, King-Holtzman genetic males with the testicular feminization (*Tfm*) mutation should lack the SNB, because such males have 85 to 90 percent fewer androgen receptors (17). Examination of the spinal cords of *Tfm* males confirmed the predicted absence of an SNB in these animals (figure 28.1c) (18).

The function of the SNB may be inferred from the function of the BC and LA, which is undoubtedly sexual since both of these muscles are attached exclusively to the penis. In other mammals the BC or its homolog is involved in male copulatory behavior (19). The muscles BC and LA are absent or vestigial in adult female rats (9). Pre- or postnatal injections of testosterone propionate (TP) are effective in masculinizing the morphology of the perineal region in females, including the LA which is present in female rats at birth, but atrophies in the first 3 weeks of life unless maintained by postnatal injections of TP (20). Such perinatal androgen injections in females also masculinize their copulatory behavior (21). Therefore, the finding that the neurons of the SNB accumulate androgens or their metabolites, but not E, together with the neonatal androgen sensitivity of the SNB's target muscles, suggest that androgens but not estrogens play a role in the development of the dimorphism of the SNB. This hypothesis is supported by the absence of the SNB in *Tfm* males with reduced androgen receptors.

The penile striated muscles are involved in reflexes discovered by Hart (22), which are controlled by the

spinal cord, and these reflexes may be related to copulatory behavior (23). Androgens, acting in the spinal cord, augment these reflexes, but E does not. Neonatal castration of males permanently reduces the frequency of such reflexes (24). The accumulation of hormone by the SNB motoneurons after androgen but not E injection suggests that the SNB is a site of action of androgens in modifying the penile reflexes. If this were the case, then the SNB cells provide significant advantages for studying the mechanisms by which a hormone modifies behavior of a vertebrate, because these neurons are large, well localized, and their behavioral function readily determined.

## References and Notes

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3. F. Nottebohm and A. P. Arnold, *Science* 194, 211 (1976).
4. R. A. Gorski, J. H. Gordon, J. E. Shryne, A. M. Southam, *Brain Res.* 148, 333 (1978).
5. See E. C. Greene [*The Anatomy of the Rat* (Hafner, New York, 1963)] or J. H. Venable [*Am. J. Anat.* 119, 271 (1966)] for drawings of the muscles injected. The BC has distinct medial and lateral portions, but as we found no differences in the location of their motoneurons, we refer to the muscles collectively as BC.
6. The anesthetic (Chloropent; Fort Dodge Laboratories) was injected intraperitoneally (0.33 ml/100 g body weight). The rats were obtained from Simonsen Laboratories.
7. Initially large (total, 15 to 50  $\mu$ l) unilateral injections were made, but retrogradely labeled cells were found bilaterally and in several locations in the cord, indicating that the HRP had spread to contralateral muscles and nearby tissue. Progressively smaller injections were made until the volume used (total, 3 to 10  $\mu$ l) produced only ipsilateral staining of a single portion of the cord.
8. The animals were killed with Chloropent and perfused, and the spinal cords were stained as in either J. S. DeOlmos [*Exp. Brain Res.* 29, 541 (1977)] or M. Mesulam [*J. Histochem. Cytochem.* 26, 106 (1978)]. Cords were sectioned in 50- $\mu$ m transverse or horizontal sections.
9. K. J. Hayes [*Acta Endocrinol.* 48, 337 (1965)] states that the LA muscle of the rat is misnamed because (i) LA is not the first name applied to this muscle, (ii) the attachments of the rat LA are not homologous to the LA of humans, and (iii) the muscle could not possibly levate the anus. Hayes suggests that the first application of the name LA to this muscle by Greene [see (5)] may have been an error, and proposes the muscle be called the dorsal bulbocavernosus. Our finding a common locus of LA and BC motoneurons lends weight to Hayes' suggestion. Other authors disagree with the newly proposed name, but agree that the term LA is erroneously applied to this muscle in the rat (20).
10. As part of another experiment (S. M. Breedlove and A. P. Arnold, in preparation) adult Sprague-Dawley male and female rats were sham castrated and injected with sesame oil vehicle for 28 days. Then the rats were killed with an overdose of Nembutal and perfused intracardially with saline, then buffered Formalin. The spinal cord was removed with the dorsal roots being used for a guide, and alternate transverse 50- $\mu$ m sections were stained with thionin. The number of nuclei of densely stained cells in alternate sections in the region described (200 to 400  $\mu$ m ventral to the central canal and within 250  $\mu$ m of the midline in L<sub>5</sub> and L<sub>6</sub>) counted by a blind observer was: males ( $N = 4$ )  $172.3 \pm 11.59$  [standard error of mean (S.E.M.)]; females ( $N = 5$ )  $46.9 \pm 11.17$ . These numbers are

corrected for split nuclei by the method of M. Abercrombie [*Anat. Rec.* 94, 239 (1946)].

11. M. Sar and W. E. Stumpf, *Science* 197, 77 (1977).

12. The hormones (from New England Nuclear) were labeled as follows—DHT: [1,2,4,5,6,7-<sup>3</sup>H]dihydrotestosterone; T: [1,2,6,7,16,17-<sup>3</sup>H]testosterone; and E: [2,4,6,7,16,17-<sup>3</sup>H]estradiol. Their specific activity was 123 to 160 Ci/mole, and doses ranged from 150 to 195  $\mu$ Ci per 100 g of body weight.

13. Kodak NTB3 emulsion was used. The autoradiographic method was a modification of that used by Pfaff and Keiner (16). For each animal, one-third of the autoradiograms was developed after one of three different intervals to provide a range of background silver densities. The mean ( $\pm$ standard deviation) background densities for the three hormones were: DHT, 6.76 ( $\pm$ 3.0); T, 12.22 ( $\pm$ 3.09); E, 10.98 ( $\pm$ 8.31) silver grains per 100  $\mu$ m<sup>2</sup>. There were no significant differences in the background density of the three hormones (one-way analysis of variance,  $P > .40$ , with N being the number of animals).

14. By this criterion one considers a cell labeled if the density of silver grains over the cell is five or more times that of the background. The relative advantages of various labeling criteria are discussed in A. P. Arnold [*J. Comp. Neurol.* 189, 421 (1980)].

15. The  $P$  value,  $<.05$ , was obtained by the two-tailed, independent t-test, with N being the number of animals and the test being done after angular transformation of the data to obtain a normal distribution [R. R. Sokal and F. J. Rohlf, *Biometry* (Freeman, San Francisco, 1969)].

16. D. W. Pfaff and M. Keiner [*J. Comp. Neurol.* 151, 121 (1973)] and D. A. Keefer, W. E. Stumpf, and M. Sar [*Proc. Soc. Exp. Biol. Med.* 143, 414 (1973)] also reported such labeling.

17. O. Naess, E. Haug, A. Ahramadal, A. Aakvaag, V. Hansson, F. French, *Endocrinology* 99, 1295 (1976).

18. Experimentally naive 109- to 115-day-old King-Holtzman males with the *Tfm* mutation and male littermates without the mutation were killed and their SNB cells counted as above (10). The *Tfm* males had a mean ( $\pm$ S.E.M.) of  $34.4 \pm 2.26$  ( $N = 7$ ) SNB cells while their normal male littermates had  $155.5 \pm 2.29$  ( $N = 7$ ) cells in this region.

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24. B. L. Hart, in *Sex and Behavior*, T. E. McGill, D. A. Dewsbury, B. D. Sachs, Eds. (Plenum, New York, 1978), p. 205.

25. We thank C. Jacobson, D. Mills, and S. Breedlove for assistance. Supported by NSF grant BNS 7705973 to A.P.A. and PHS grant 5-S07 RR07009-14 to the University of California, Los Angeles.

In the male rat, the bulbocavernosus (BC) and levator ani (LA) muscles are innervated by about 200 motoneurons which can be readily distinguished as a nucleus in the lumbar spinal cord. This spinal nucleus of the bulbocavernosus (SNB) is not discernible as a nucleus in female rats because the densely staining neurons in this region are only about one-third as numerous and one-half as large as those in the male (Breedlove and Arnold, 1980, 1981). Similarly, the target muscles of the SNB are absent in normal adult females. The motoneurons of the adult male SNB accumulate radioactivity following injections of tritiated testosterone (T) or dihydrotestosterone (DHT), but not estradiol (E) (Breedlove and Arnold, 1979).

The sexually dimorphic appearance of the SNB and the hormone accumulation by SNB neurons in adulthood suggest that perinatal androgens may be responsible for the sexually dimorphic development of this system. This conjecture is confirmed by the markedly feminine appearance of the SNB in male testicular feminized (*tfm*) rat mutants which have a smaller number of androgen receptors (Naess et al., 1976; Fox, 1975). Further support for the role of androgens in SNB masculinization is found in the increased number of SNB neurons in adult female rats when they are treated perinatally with testosterone propionate (TP) or dihydrotestosterone propionate (DHTP) (Breedlove and Arnold, 1983), but not when treated neonatally with estradiol benzoate (EB) (Breedlove et al., 1982).

The present experiments were designed to address further the hypothesis that androgens guide the masculinization of the SNB, specifically by analyzing the effects of the nonsteroidal anti-androgen, flutamide (FL; Neri et al., 1972), on the SNB of male rats. Prenatally administered FL has been reported to cause deficits in the copulatory behavior of male rats (Clemens et al., 1978). There were three principal reasons for attempting to manipulate the appearance of the SNB with FL: (1) FL demasculinization of the SNB would provide evidence that estrogen receptors do not play a role in the masculinization of this nucleus, since FL is not known to interfere with estrogen receptors; (2)

FL demasculinization of the SNB system would also indicate that androgen stimulation is necessary for the masculinization of the SNB, even in genetically normal males; and (3) the ability to demasculinize the SNB with a treatment such as FL would provide a valuable research tool complementing the ability to masculinize the SNB with androgens.

The present results show that when given prenatally, FL very successfully prevents the masculinization of the SNB and its target muscles but does not interfere with the conventional measures of sexual behavior in male rats. From these results we infer that androgens, but neither estrogens nor any nonhormonally mediated genetic mechanisms, are crucial for the normal male development of the SNB system.

#### Materials and Methods

After female rats displayed at least two regular 4-day cycles, they were left in a group cage with males on the day of proestrus. If sperm remnants were seen in the vaginal smear the following day, this day was designated as day 1 of gestation. From day 1 until day 10 of gestation, pregnant dams were gently handled each day. Rat fetuses were treated prenatally with flutamide by injecting the pregnant dam daily with 5 mg of flutamide in 0.1 ml of 1,2-propanediol (propylene glycol; PG), subcutaneously. This treatment was given from day 11 of gestation up to and including the day before birth. The dams of control pups were injected with PG vehicle only. All dams delivered normally on day 23 of gestation (day 1 postnatal). On the day of birth the ano-genital distance (AGD) of each pup was measured. In addition, all pups were castrated and identified individually by means of toe clipping. Because prenatal FL treatment did not alter the appearance of the gonads at birth, day 1 castration provided the opportunity to determine the sex of the animals. This method of sex determination was necessary because prenatal FL greatly reduced the AGD of males, making male and female AGDs indistinguishable. Some female pups were sacrificed, leaving each dam with eight pups.

It is possible that dams treated with flutamide might release the anti-androgen in their milk supply following delivery. This potential contamination could obscure independent manipulation of pre- and postnatal hormonal factors. In order to detect such a continued action of flutamide postnatally, one-half of the animals from each group were cross-fostered. For example, one-half of the pups exposed to flutamide prenatally were cross-fostered on the day of birth to a dam that had received propylene glycol (PG) vehicle and had that same day delivered a litter of prenatal PG pups, one-half of which were cross-fostered to a dam that had received flutamide. Beginning on the day of birth and on each alternate day until postnatal day 11, pups were injected with either 1 mg of testosterone propionate (TP) in 0.05 ml of sesame oil, s.c., or oil vehicle alone. When pups were injected, the needle puncture was sealed with flexible collodion. Thus the final experimental design consisted of eight groups of male pups in a simple three-way analysis of variance with the following factors: prenatal treatment (either flutamide or PG vehicle), postnatal treatment (either TP or oil vehicle), and fostering condition (either cross-fostered or left with the natural mother).

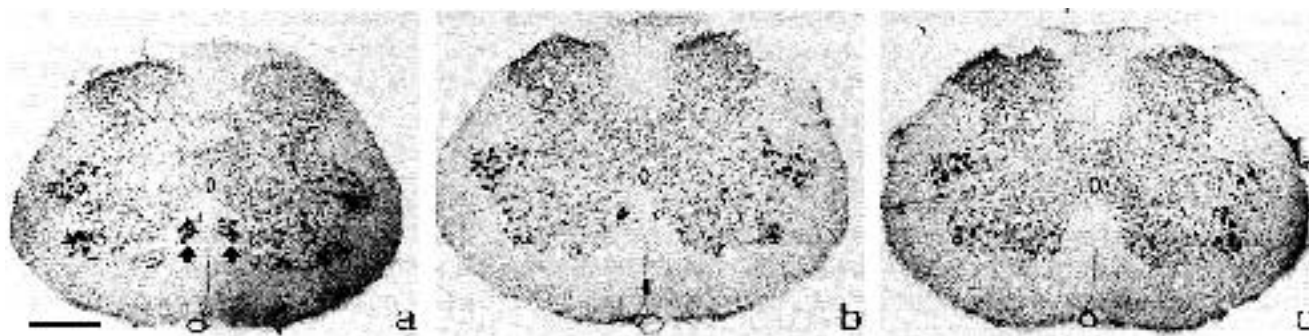
All pups were weaned on day 21 of life and housed in same-treatment groups of 3 to 4. In order to equalize the activational influence of androgens, all rats were given daily injections of TP (2 mg/kg, s.c.) in a sesame oil vehicle (5 mg of TP/ml) from day 68 of life until sacrifice on days 101 to 103. The masculine copulatory behavior of all animals was tested beginning 22 days after androgen treatment commenced. Each animal was tested in four sessions, with 2 days of rest between each. Test sessions were conducted during the dark portion of the light cycle, in the room where animals were raised and housed. Each session consisted of leaving the test animal alone in a glass aquarium (30 × 60 × 40 cm high) for 10 min, then introducing a hormonally primed, receptive stimulus female, which was replaced by another receptive female after 10 min. Ten minutes after the introduction of the second stimulus female, the session was ended. Masculine copulatory behavior of the test animals was measured by the postural correlates of mounting, intromission, and ejaculation. A mount was counted if it was accompanied by pelvic thrusting, but not intromission. An intromission was defined as a mount ending with a springing, backward dismount. Ejaculation was defined as a mount followed by a rearing and flexion of the forelimbs, usually accompanied by a high-pitched vocalization. In normal males these behaviors are quite unmistakable and have been correlated with literal intromission of the penis and ejaculation of semen. In addition to measuring the number of such behaviorally defined mounts, intromissions, and ejaculations, the

latencies for each were measured. If an animal failed to show a particular behavior, a maximal latency of 1200 sec was assigned for that session. The ratio of the number of mounts with intromission compared with the total number of mounts was computed as "intromission efficiency." For those animals displaying ejaculation, the post-ejaculatory interval before the next intromission and the inter-ejaculatory interval were measured. The stimulus females were made responsive with subcutaneous injections of 20  $\mu$ g of estradiol benzoate approximately 54 and 30 h prior to the test session, and a single injection of 0.5 mg of progesterone 6 h before the test.

Animals were sacrificed with sodium pentobarbital and were perfused intracardially with saline followed by buffered 10% formalin. The spinal cords from lumbar segment 4 to sacral segment 1 were removed and postfixed at least 1 week in buffered formalin. The perineal muscle complex from each animal, consisting of the muscles LA, BC, and ischiocavernosus and the base of the penis to which these muscles attach, was also removed and postfixed.

Spinal cords were frozen-sectioned at 50  $\mu$ m and alternate sections were mounted and stained with thionin. A "blind" observer then counted the number of nuclei of densely staining cells in the region occupied by the SNB in normal males: 200 to 400  $\mu$ m below the central canal, within 250  $\mu$ m of the midline in lumbar segments 5 and 6. A dozen of these neurons were chosen at random and their somas were traced through a camera lucida. The mean area of these 12 cell somas provided a single score for each animal for analysis. This sampling procedure provides a quite sensitive measure of somatic and nuclear profile size, since analysis of variance detected highly significant effects of all three manipulations on somatic area (see figure 29.3, results below). The area of the nuclei provided an estimate of the mean nuclear diameter for use in Konigsmark's (1970) formula to correct for split nuclei error. This correction procedure is more fully described in Breedlove and Arnold (1981).

After several weeks of fixation, the perineal muscle complex from each animal was briefly dried with a paper towel to remove excess formalin, and then weighed. Additionally, a sample of the levator ani was removed. The number of LA fibers was taken to be representative of the total number of SNB target fibers, i.e., of both the LA and BC, because of the following considerations. The architecture of the BC fibers makes estimation of their numbers difficult. However, the presence or absence of the BC and LA muscles in individuals in different groups was perfectly correlated, and the estimate of LA fibers was correlated with the overall perineal muscle complex weight ( $r = +0.6$ ,  $p < 0.001$ ,  $N = 31$  rats with muscles). The LA sample



**Figure 29.1**

Photomicrographs of thionin-stained sections from the fifth lumbar segment of the adult rat spinal cord in the region of the spinal nucleus of the bulbocavernosus (SNB). *a*, Transverse section of the spinal cord of a male exposed to vehicle prenatally, castrated at birth, but given TP therapy just after birth. The arrows point to the SNB, which is morphologically identical to that of a normal male (e.g., see Breedlove and Arnold, 1983, figure 29.1*a*). *b*, Transverse section from a male given the anti-androgen flutamide prenatally, but given TP postnatally. Such males have fewer, smaller SNB cells and fewer target muscle fibers than do control males. *c*, Males given flutamide prenatally, castrated at birth, and given oil postnatally have spinal cords that are indistinguishable from those of normal females. Additionally, 9 of 10 such males, like normal females, totally lack the SNB target muscles. Scale bar, 500  $\mu$ m; sections are 50  $\mu$ m thick.

was stained overnight with 2% osmium tetroxide, dehydrated in alcohol, cleared in acetone, and then embedded in a soft epoxy resin plastic mixture. This mixture consisted of 110 parts dodecenylsuccinic anhydride, 65 parts Poly/Bed 812 (Polysciences, Inc., Warrington, PA), and 3 parts 2,4,6-tri-(dimethylaminomethyl)phenol. The plastic embedded muscles were left in a 58°C oven overnight to harden and then were sectioned 20  $\mu$ m thick in the plane perpendicular to the fibers, using a sliding microtome. The sections were mounted on microscope slides using fresh resin mixture which was then hardened in an oven overnight. To estimate the number of fibers in the LA sample, the total area of the LA cross-section was divided by the mean area of 24 randomly sampled individual fibers. This estimate represents the number of LA fibers on one side and is valid as long as there is no branching of LA muscle fibers. We found no evidence of such branching since, for several animals, the total number of muscle fibers was counted from three locations in the longitudinal extent of the LA and were found to be quite comparable to estimates calculated as above. The number of muscle fibers appeared constant over the length of the muscle. Additionally, several LA muscles were cut longitudinally, but no evidence of fiber branching was seen.

Statistical analysis consisted of simple three-way analyses of variance (prenatal treatment  $\times$  postnatal treatment  $\times$  fostering condition). This analysis was applied to the following measures: number of SNB neurons, cross-sectional area of SNB somas, cross-sectional area of SNB neuronal nuclei, weight of perineal muscle complex, number of LA fibers on one side, and mean number of mounts, intromissions, and ejaculations. This analysis was also applied to the intromission efficiency and mean latencies for the three

copulatory behaviors, following an angular transformation to approximate a normal distribution (Sokal and Rohlf, 1969, p. 386). For this purpose, latencies were converted to proportions of the maximum latency (1200 sec). Five rats from each of the eight groups were randomly chosen for morphological and behavioral analyses. Rats from at least three different litters made up the five subjects for each group.

## Results

### Number of SNB Neurons

Prenatal flutamide treatment of male rats resulted in significantly fewer SNB neurons ( $p < 0.001$ ) compared to PG-treated controls. Postnatal TP treatment resulted in significantly more SNB cells than did oil treatment ( $p < 0.001$ ), although this postnatal treatment could not completely overcome the demasculinizing effects of prenatal flutamide exposure (figures 29.1 and 29.2). Because there was no significant effect of cross-fostering on SNB neuron number, figure 29.2 represents the mean number of SNB cells in the groups combined across fostering conditions. There was a significant interaction between pre- and postnatal treatments ( $p < 0.01$ , figure 29.2), apparently because postnatal TP had a much greater effect on rats that had been treated prenatally with flutamide.

### SNB Soma Cross-Sectional Area

Prenatal flutamide resulted in smaller SNB soma areas ( $p < 0.001$ ), while postnatal TP led to an increased SNB soma size ( $p < 0.001$ ). Again, there was a significant interaction between pre- and postnatal treatments ( $p < 0.001$ ), which is displayed in figure 29.3. There was a significant main effect of cross-fostering on SNB soma size ( $p < 0.01$ ), but there were no significant

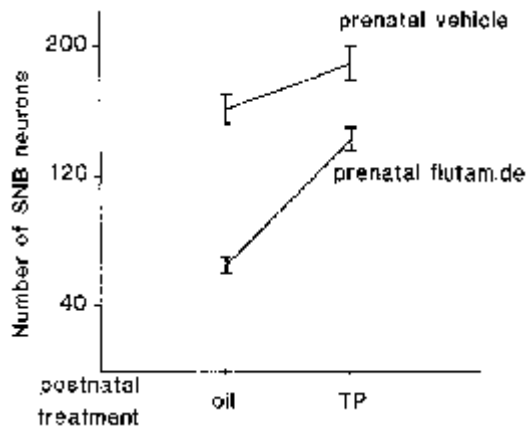


Figure 29.2

Prenatal treatment with the anti-androgen, flutamide, significantly demasculinizes the number of SNB motoneurons in male rats. Postnatal treatment with TP partially compensates for this demasculinization. All rats were castrated on the day of birth. Males treated prenatally with flutamide and given only oil postnatally have about as few SNB neurons ( $65.0 \pm 4.8$ , lower left on graph above) as do control females ( $66.6 \pm 4.5$ , not depicted above).

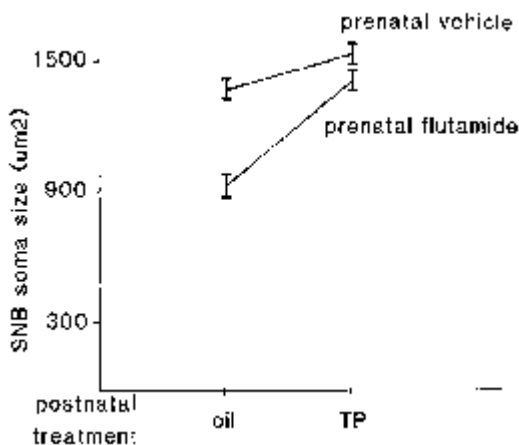


Figure 29.3

Prenatal treatment with the anti-androgen, flutamide, significantly demasculinizes the soma size of SNB motoneurons in male rats. Postnatal TP treatment partially compensates for this demasculinization. All rats were castrated on the day of birth and received TP treatment in adulthood prior to sacrifice.

interactions of fostering conditions with any other factor. Cross-fostering resulted in larger SNB somas regardless of whether the pups were fostered by a dam that had received flutamide or PG vehicle. However, since there were no interactions of cross-fostering with any other factor, figure 29.3 displays soma areas collapsed across fostering conditions.

#### SNB Neuronal Nuclei Cross-Sectional Area

The size of the nuclei of SNB neurons was smaller following prenatal FL ( $p < 0.001$ ) and larger after postnatal TP ( $p < 0.001$ ), but there was no significant interaction of the pre- and postnatal treatments (table

Table 29.1

The cross-sectional area of the nuclei of SNB neurons of adult male rats treated prenatally with the anti-androgen, flutamide, or PG vehicle

	Postnatal Treatment	
	Oil	TP
$\mu\text{m}^2$		
Prenatal flutamide	$162.0 \pm 9.89$	$215.1 \pm 8.07$
Prenatal PG vehicle	$226.8 \pm 7.04$	$251.0 \pm 13.04$

All pups were castrated on the day of birth. Postnatally, pups received either TP or oil vehicle. Standard errors of the mean are given, based on  $N = 10$  animals per group.

Table 29.2

The mean weight ( $\pm$  standard error of the mean), in grams, of the perineal muscle complex from adult male rats castrated on the day of birth

	Postnatal Treatment			
	Oil		TP	
	Cross-Fostering	Natural Fostering	Cross-Fostering	Natural Fostering
Prenatal FL	0 (0)	0.10 (0.1)	1.07 (0.05)	1.03 (0.06)
Prenatal PG	1.20 (0.05)	1.03 (0.05)	1.78 (0.03)	1.59 (0.08)

Prenatal treatment with the anti-androgen, flutamide (FL), decreased the perineal muscle weight, compared to rats prenatally treated with propylene glycol (PG) vehicle. Postnatal TP increased muscle weight, especially in rats treated with FL prenatally. Half of the pups treated prenatally with flutamide were cross-fostered on the day of birth to dams that had received PG injections. Similarly, half of the pups treated prenatally with PG were cross-fostered to flutamide-treated dams. Other pups were left with their natural mothers. Cross-fostering significantly increased muscle weight only in animals prenatally treated with the PG vehicle.  $N = 5$  animals per group.

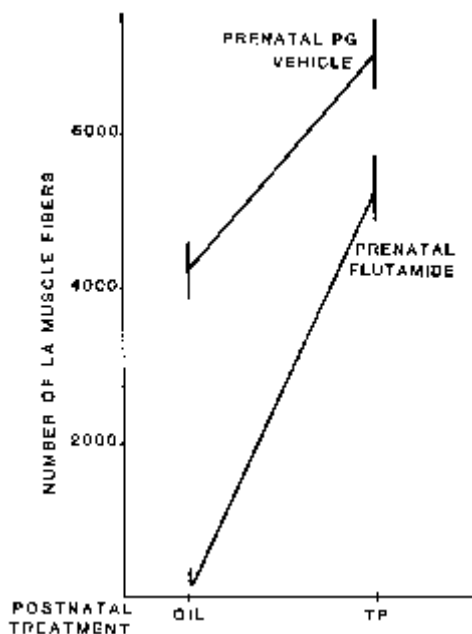
29.1). There were no significant effects of cross-fostering.

#### Perineal Muscle Weight

Prenatal flutamide decreased perineal muscle complex weight ( $p < 0.001$ ), and postnatal TP increased muscle weight ( $p < 0.001$ ; table 29.2). TP had a significantly greater masculinizing effect on animals that had received prenatal flutamide rather than prenatal PG ( $p < 0.001$ ). While cross-fostering did not have a significant main effect on muscle weight, there was a significant interaction of fostering and prenatal treatment ( $p < 0.05$ ). Specifically, cross-fostering resulted in increased muscle weight of animals receiving prenatal PG ( $p < 0.05$ ) but did not alter the muscle weight of animals treated with flutamide prenatally ( $p > 0.05$ ; table 29.2).

#### Number of LA Muscle Fibers

The estimated number of LA muscle fibers was decreased by prenatal FL ( $p < 0.001$ ) and increased



**Figure 29.4**

The number of fibers in the muscle levator ani (LA) is significantly reduced by prenatal treatment of male rats with the anti-androgen, flutamide. In fact, only 1 of the 10 males receiving prenatal flutamide and postnatal oil had the SNB target muscles in adulthood. Postnatal TP treatment increases the number of LA fibers. Postnatal TP treatment had a greater effect in animals receiving prenatal flutamide.

by postnatal TP ( $p < 0.001$ ; figure 29.4). Once again, postnatal TP had a significantly more masculinizing effect on animals treated with prenatal FL ( $p < 0.01$ ). There were no significant effects of cross-fostering on the number of LA fibers.

#### Masculine Copulatory Behavior

Neither the frequency nor latency of mounting behavior was significantly affected by either prenatal or postnatal androgen manipulation ( $p > 0.05$ , three-way analysis of variance, data not shown). Intromission frequency (table 29.3) and latency were altered by postnatal TP treatment ( $p < 0.02$ ) but were not affected by prenatal FL exposure ( $p > 0.05$ ). There were no significant interaction effects or effects of cross-fostering on either mounting or intromission. Four of the animals exhibited ejaculatory behavior once each. Three of these rats were treated with vehicle prenatally and TP postnatally, and were left with their biological mother. The fourth animal received flutamide prenatally and TP postnatally, and was cross-fostered at birth. Thus, while there was a significant three-way interaction of prenatal, postnatal, and fostering conditions on the number of ejaculations ( $p < 0.02$ ), no single factor exhibited a predominant influence on ejaculation under the present testing conditions.

**Table 29.3**

The mean number of intromissions displayed per 20-min test session by male rats treated prenatally with flutamide or vehicle

	Neonatal Treatment	
	Oil	TP
Prenatal flutamide	$2.2 \pm 0.58$	$5.7 \pm 1.85$
Prenatal vehicle (PG)	$2.0 \pm 0.63$	$4.8 \pm 1.19$

All pups were castrated at birth and treated with either TP or oil neonatally. The rats also received TP treatment in adulthood before and during testing. Analysis of variance revealed no effect of prenatal flutamide treatment or neonatal cross-fostering on intromission behavior. Neonatal TP treatment, however, significantly augmented intromission.

#### Fetus and Pup Survival

There was no appreciable mortality caused by either the prenatal or postnatal treatments. All FL- and PG-treated dams delivered live pups with a mean of 10.2 pups per FL litter and 12.3 pups per PG litter. Following live birth and day 1 manipulations, only 2 of 27 PG-treated pups and 3 of 32 FL-treated pups died before sacrifice on days 101 to 103 of life. On the day of birth, the AGD of males exposed to prenatal FL was  $2.61 \pm 0.07$  (SEM,  $N = 20$ ) mm, while that of males exposed to prenatal PG vehicle was  $4.57 \pm 0.06$  ( $N = 20$ ). The AGD on the day of birth of females exposed to prenatal FL was  $2.43 \pm 0.08$  mm ( $N = 14$ ), while that of those exposed to prenatal PG was  $2.55 \pm 0.04$  mm ( $N = 18$ ).

#### Discussion

The anti-androgen flutamide significantly demasculinized the SNB and its target muscles. Prenatal flutamide resulted in fewer SNB neurons, which had smaller somas, smaller neuronal nuclei, and fewer target muscle fibers. Postnatal TP treatment, on the other hand, resulted in significantly more SNB neurons which had larger somas, larger nuclei, and more target muscle fibers. The demasculinization of the SNB system by anti-androgen, the masculinizing effect of androgens in the present and other studies (Breedlove and Arnold, 1983; Breedlove et al., 1982), and the feminine appearance of the SNB in *tfm* rats (Breedlove and Arnold, 1981) leave little doubt that androgens are normally responsible for the sexually dimorphic development of the SNB system.

There was also significant interaction between the pre- and postnatal treatment effects on virtually every measure of the SNB system, including SNB cell number, SNB soma size, perineal muscle weight, and number of LA muscle fibers. The interaction consisted of a much more pronounced masculinizing effect of TP when given to flutamide-treated rather than vehicle-

treated pups. This result implies that the SNB system of a rat receiving normal male levels of androgen prenatally is not very sensitive to postnatal androgen deprivation. Conversely, a rat receiving little prenatal androgen exposure, such as a normal female or a male deprived of androgen action by prenatal flutamide, *would* be sensitive to TP treatment immediately after birth. Thus, androgens present during either the prenatal or postnatal period can significantly compensate for a lack of androgens during the other period. This compensatory hypothesis is supported by two other findings. First, day 1 castration of male rats caused a statistically nonsignificant loss of SNB neurons, but treatment of female rats with DHTP just after birth caused a significant increase in the number of SNB neurons seen in adulthood (Breedlove and Arnold, 1983).

Previous studies have implied that the aromatized metabolite of testosterone, estradiol (E), is primarily responsible for masculinization of the brain (McEwen et al., 1977). For example, E alone is effective in masculinizing the pattern of gonadotropin release (Gorski and Wagner, 1965), lordosis behavior (Christensen and Gorski, 1978), and masculine copulatory behavior (Sodersten and Hansen, 1978). Furthermore, T is known to convert readily to E in the rat CNS (Naftolin et al., 1975). However, there is now a good deal of evidence that androgens per se guide the masculine development of the SNB without requiring aromatization. First, male *tfm* rats with normal levels of brain E receptors and subnormal levels of brain androgen receptors (Olsen and Fox, 1981) have a markedly feminine SNB (Breedlove and Arnold, 1981). Second, a single injection of TP (1 mg), but not EB (100  $\mu$ g), on day 2 of life masculinized the number of SNB cells in adult females (Breedlove et al., 1982). Third, the non-aromatizable androgen, DHTP, when delivered immediately after birth, leads to a greater number and size of SNB motoneurons (Breedlove and Arnold, 1983). Finally, the present demonstration that prenatal flutamide demasculinizes the SNB suggests that estrogen receptors do not play a role in the development of the nucleus since flutamide is ineffective in blocking tritiated E uptake in rat uterine extract (DeBold et al., 1981; J. DeBold, personal communication). On the other hand, flutamide effectively blocks DHT uptake by receptors in the rat kidney and brain (DeBold et al., 1981). Hence the demasculinization caused by flutamide or its metabolites is probably due to interference with androgen but not estrogen action.

Inasmuch as prenatal FL has drastic consequences for the development of the SNB system, one may infer that those behaviors altered by FL treatment may involve this neuromuscular complex, and those behaviors

unaffected by FL probably do not involve the SNB system. Therefore, traditionally defined mounting and intromission behaviors, which in the present study were unaffected by FL treatment, are probably not mediated by the SNB or its target muscles. In fact, all of the males receiving FL prenatally and oil postnatally displayed some mounting and intromission behavior, even though the majority of these males lacked the SNB target muscles. Since the SNB target muscles attach exclusively to the base of the penis, the SNB neurons undoubtedly control functions of the penis. However, such control need not be reflected in the gross postural movements associated with intromission in normal males and which, for convenience, are often equated with literal intromission of the penis. For example, the postural correlates of intromission are displayed by normal females given TP in adulthood (Ward, 1969), but these females do not possess a penis or the associated striated perineal muscles, and they are clearly not intromitting in a typically male fashion. Similarly, *tfm* male rats occasionally show intromission and even ejaculation-like behavior patterns (Olsen, 1979), even though they lack the SNB target muscles (Breedlove and Arnold, 1981). But if the BC/LA muscles are not essential for the display of the postural correlates of intromission, there is direct evidence of their importance to male fertility (Sachs, 1982) and penile reflexes (Hart and Melese-d'Hospital, 1981; Sachs, 1982). In other species, the BC muscle is active during literal erection and/or ejaculation (Kollberg et al., 1962; Hart, 1972; Beckett et al., 1975).

While the present results deny the importance of aromatized metabolites for the masculinization of the SNB system, the effectiveness of TP in masculinizing but the ineffectiveness of FL in demasculinizing mounting and intromission implies that aromatized androgens may be important for those functionally defined behaviors. It may be that FL has no effect on these behaviors because estrogen receptors within the CNS are not blocked by FL and are responsible for the masculinization of these behavioral correlates. On the other hand, it is still possible that androgen receptors contribute to the masculinization of these behaviors since Clemens et al. (1978) reported fewer mounts and intromissions by male rats given prenatal FL. The discrepancy between the results of Clemens et al. (1978) and the present study may be due to differences in the strains of rats used, dose of TP in adulthood, or age at which the males were castrated.

It appears that the sex differences in the SNB can be totally explained by androgen-induced changes during the perinatal period and that other, nonhormonally mediated mechanisms need not be involved. Such an idea was also suggested when *tfm* rats were found to

have a feminine SNB system (Breedlove and Arnold, 1981). Since the only known defect in these animals is in their androgen receptors (Naess et al., 1976; Fox, 1975), it appeared that the only aspects of the genome affecting the SNB were those affecting hormonal action. Nonetheless, it is theoretically possible that there are other, nonhormonally related defects in the *tfn* genome which might be responsible for the feminine SNB. This possibility is made less likely by the present demonstration that hormonal manipulation by prenatal FL treatment combined with neonatal castration can completely demasculinize the SNB of rats with a normal male genotype.

The cross-fostering was intended to detect any postnatal demasculinization that might be caused by contamination of the milk supply of the dams treated with flutamide prior to delivery. We found no evidence of such contamination. Most measures, i.e., the number of SNB cells, SNB neuronal nuclear size, and number of LA fibers, were unaffected by cross-fostering. Unexpectedly, the two measures that were altered by cross-fostering actually resulted in a slight masculinization of PG-treated pups cross-fostered to a FL-treated dam. Specifically, SNB soma size is masculinized by cross-fostering regardless of the treatment of pups or dams. Perineal muscle complex weight was increased only in PG-treated pups cross-fostered to FL-treated dams. Therefore the relatively weak effects of cross-fostering are probably due to cross-fostering per se, independent of FL or PG treatment of the dam. Thus any flutamide leakage to a dam's milk supply is of little consequence to the masculinization of the SNB system. The fact that cross-fostering itself altered SNB soma size but not the number of SNB neurons implies that there are independent mechanisms for the masculinization of these two dimorphic aspects of the SNB (see Breedlove and Arnold, 1983).

The above results demonstrate that the SNB system is hormonally sensitive during both the prenatal and postnatal periods. Perinatal treatment of females with androgens also affirms this hormone sensitivity (Breedlove and Arnold, 1983). The mechanism whereby androgens masculinize the number of SNB neurons is presently unknown. However, the fact that postnatal manipulation can reduce the number of SNB cells in males may rule out the possibility that hormones alter proliferation of these neurons, since recent experiments in this laboratory have shown that male SNB cells are postmitotic by day 14 of gestation (Breedlove, et al., 1983). Similarly, the site at which androgen masculinizes the SNB may be the target muscles, which have androgen receptors in adulthood (Dube et al., 1976), or the SNB motoneurons themselves, which accumulate androgens (Breedlove and Arnold, 1980) and respond

morphologically to TP in adulthood (Breedlove and Arnold, 1981).

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Sex differences in the behavior of vertebrates are ubiquitous and have long been recognized, but only recently have sexual dimorphisms been identified in central nervous system (CNS) areas mediating sexually dimorphic behaviors. These anatomical differences can be striking and include dimorphism in the type and number of synapses and large regional sex differences in cell size or number (1, 2). In several cases sexual dimorphisms in CNS morphology have been shown to develop under hormonal control. For example, the sex difference in the size of nuclei involved in copulatory behavior of rats and sexually dimorphic song production in zebra finches is diminished by treating females with androgen during early development (3, 4). Neonatal androgen treatment of female rats also attenuates the dimorphism in the sexually dimorphic nucleus of the preoptic area and in the pattern of connectivity in the preoptic area (5, 6).

Until quite recently, the question of whether similar differences exist in the CNS of humans has not been addressed. Swaab and Fliers (7) reported a sexual dimorphism in the volume and cell number of a nucleus in the preoptic area of humans, comparable to findings in rats. The function of the sexually dimorphic portion of the preoptic area has not been established in either species (8), although surrounding neural areas in rats are involved in the regulation of male sexual behavior and cyclic release of gonadotropins (9). In the present study we have identified a sex difference in humans and dogs in a spinal nucleus known to be involved in copulatory behavior.

Onuf's nucleus, in the ventral horn of the sacral spinal cord, is morphologically very similar in humans, monkeys, cats, and dogs (10–14). It consists of a slender column of small-to-medium-sized motoneurons that stands out sharply in myelinstained coronal sections as a pale area, or "halo," demarcated from surrounding myelinated fibers. The halo apparently results from an intense concentration of longitudinally running dendrites from motoneurons within the nucleus (15).

Human neurological studies and retrograde labeling studies in cats and dogs (11–14, 16, 17) show that Onuf's nucleus innervates striated perineal muscles

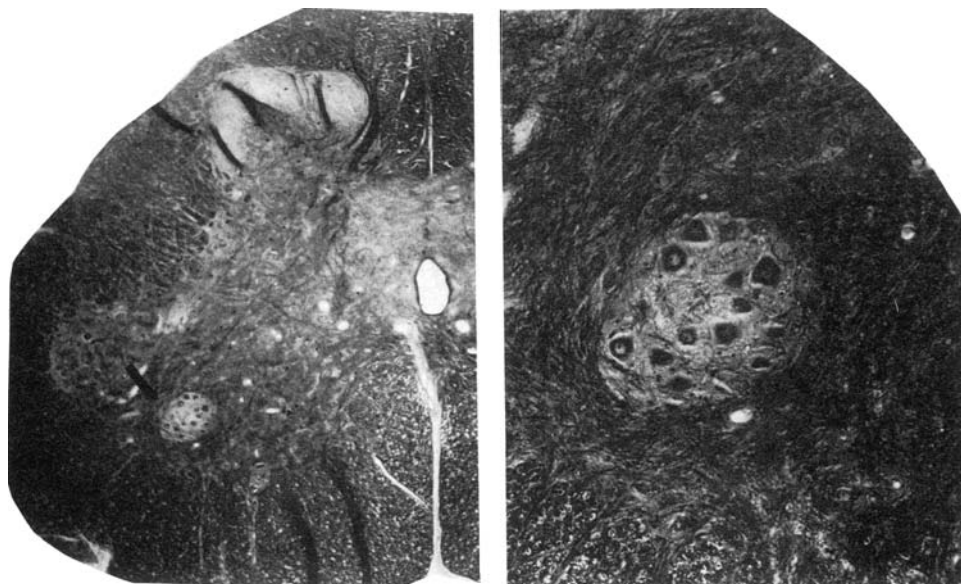
including the bulbocavernosus (BC) and ischiocavernosus (IC) muscles as well as the external anal and urethral sphincters. The BC and IC muscles attach to the penis in males and play important roles in erection and ejaculation in humans, and penile reflexes in dogs and rats (18–21). In female dogs and humans, the IC muscle is considerably smaller than in males and the BC forms circular muscles serving to constrict the vagina (22, 23). Contractions of the BC homologue have been recorded in female dogs during copulation and genital stimulation (24). Here we describe a sex difference in the number of motoneurons comprising Onuf's nucleus of humans and dogs and report further that the canine dimorphism can be eliminated by treating females with androgen early in development.

## Materials and Methods

### Dogs

Spinal cords and perineums were obtained from adult purebred beagles that had served as subjects in behavioral studies unrelated to the current report (25). Normal females ( $n = 4$ ) and males ( $n = 4$ ) were compared with five androgenized females born to dams that received daily injections of testosterone propionate (1.1 mg/kg of dam's body weight) during days 24–43 postcoitum. Four of these females also received a 37.5-mg subcutaneous implant of crystalline testosterone on the day of birth. The implant was placed dorsally beneath the skin of the neck and was gradually resorbed during the first 30–40 postnatal days.

The dogs were sacrificed as adults (3–6 yr) and perfused with saline and formalin. Perineums were dissected and examined for muscular morphology. Spinal cords were embedded in paraffin, sectioned at 15  $\mu$ m, and treated with a Klüver–Barrera stain. Bilateral counts in every third section were made of all motoneurons within the Onuf's nucleus halo. The resulting sum for each dog was tripled to estimate the total number of Onuf's nucleus motoneurons. Counts were corrected for split somas by the method of Königsmark (26). In addition, six camera lucida tracings were made at set intervals through each dog's cord of the halo defining Onuf's nucleus; mean cross-sectional halo area



**Figure 30.1**

Coronal Klüver-Barrera-stained section through Onuf's nucleus in the sacral spinal cord of a female dog exposed to androgen early in development. (A) The arrow points to Onuf's nucleus, which stands out as a pale, oval area devoid of myelinated fibers. (B) Higher-power magnification of the nucleus in A.

and the total volume of Onuf's nucleus were determined from these tracings. Somas of motoneurons in 10 representative halos from each dog were also drawn to estimate mean soma size. All counts and measurements were made by an experimenter "blind" to group membership of the sections being examined.

### Humans

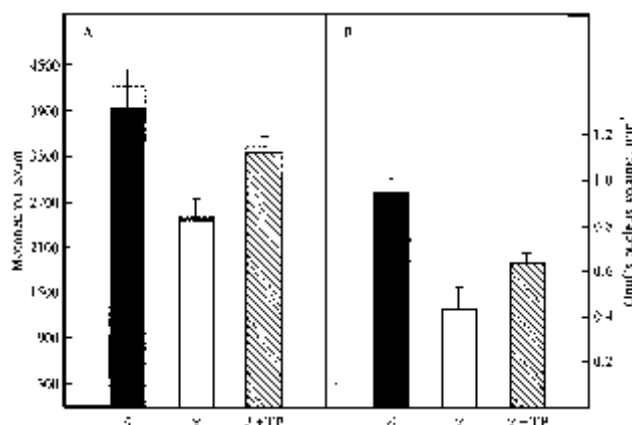
Spinal cords of eight human females and nine males from the Yakovlev collection of the Armed Forces Institute of Pathology were examined. Subjects died of causes unrelated to spinal cord function; ages at autopsy ranged from 2 to 87 yr in females (mean  $\pm$  SEM,  $22 \pm 10$  yr) and 1.5 mos to 60 yr in males (mean  $\pm$  SEM,  $22 \pm 8$  yr). Each cord was embedded in celloidin and cut coronally at  $35 \mu\text{m}$ ; and every 5th ( $n = 1$ ), 10th ( $n = 10$ ), or 20th ( $n = 6$ ) section was mounted and Nissl-stained. The sampling ratio was well matched between sexes. Adjacent Weigert-stained sections aided nucleus identification.

Motoneurons comprising Onuf's nucleus were counted in Nissl-stained sections, and the sum was adjusted according to the sampling ratio to estimate total motoneuronal number.

### Results

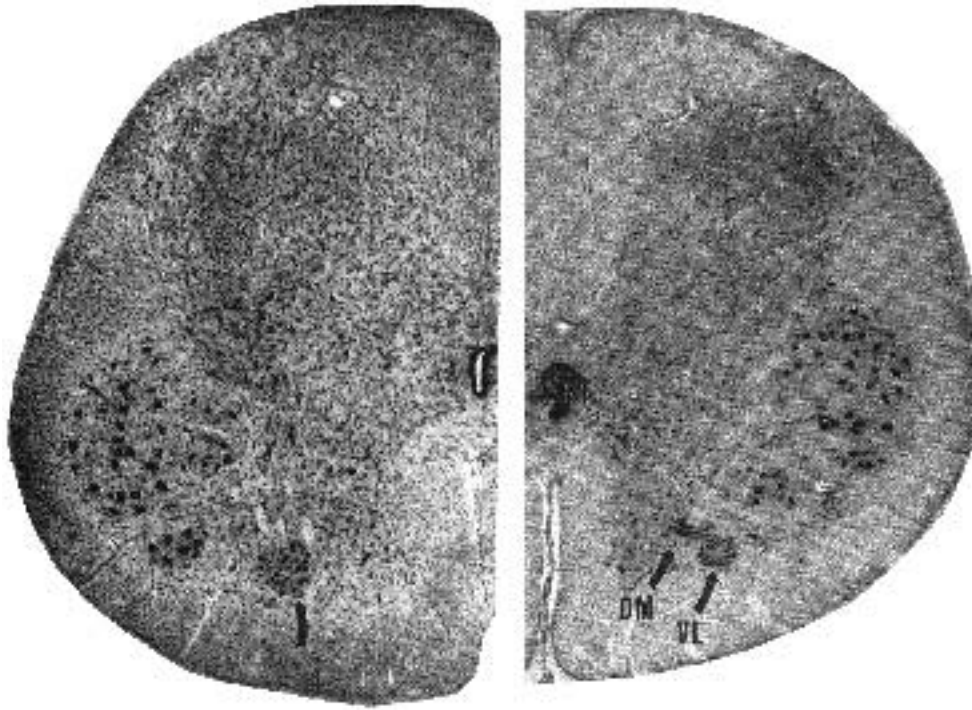
#### Dogs

Figure 30.1 depicts canine Onuf's nucleus in which both halo and individual motoneurons are clearly visible. Male dogs had significantly more neurons in



**Figure 30.2**

The number of motoneurons in Onuf's nucleus (A) and the bilateral volume of Onuf's nucleus (B) in male, female, and androgen-treated female dogs. Bars represent means  $\pm$  SEM. Dashed lines in A indicate uncorrected counts, while solid lines are counts corrected for split somas. Females had significantly fewer motoneurons and smaller nuclear volumes than did males. Females treated with androgen early in development did not differ significantly from males in motoneuron number and had more neurons and larger nuclear volumes than did untreated females. TP, testosterone propionate.

**Figure 30.3**

Nissl-stained sections through Onuf's nucleus in humans. The arrow in *A* points to VL Onuf's nucleus; no DM cells were observed in this section. Both VL and DM cell groups can be identified in *B*.

Onuf's nucleus than did females ( $P < 0.02$ , two-tailed  $t$  test) (figure 30.2A). Onuf's nucleus of androgenized females was masculinized; motoneuron number was not different from that of males ( $P > 0.25$ ) and was significantly greater than the number of motoneurons in normal females ( $P < 0.05$ ). These differences were observed for raw motoneuronal counts and for counts corrected for split somas. Perineal musculature of androgen-treated females also was markedly masculinized: external genitalia consisted of a penis with BC and IC muscles surrounding the base of the phallus, as in males. Masculinization of the perineum and spinal cord of the androgenized female receiving testosterone propionate prenatally only was as complete as for the four females receiving testosterone at birth in addition to the prenatal treatment. Sexual differentiation of the CNS probably occurs prenatally in dogs; postnatal testosterone treatment was initiated for unrelated behavioral studies (25) and apparently did not influence spinal organization.

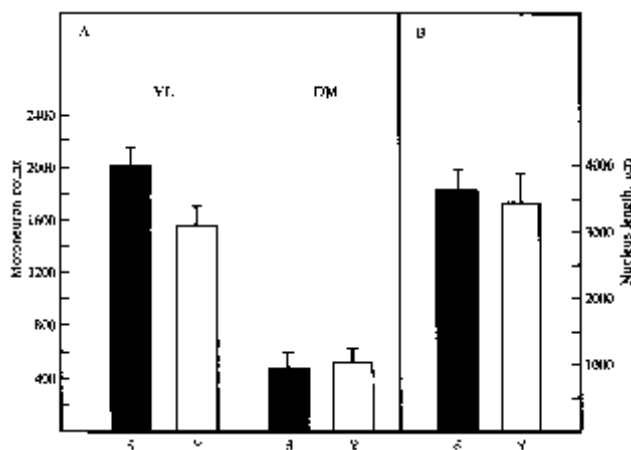
The total bilateral volume of Onuf's nucleus (mean cross-sectional area  $\times$  the length of the nucleus) was 2.2 times greater in male dogs than in female dogs ( $P < 0.01$ ) (figure 30.2B). The volume of Onuf's nucleus in androgenized females was intermediate and differed significantly from both males and untreated females ( $P < 0.05$ ). Mean motoneuronal soma size was not significantly different in the three groups.

### Humans

Onuf's nucleus in humans is divided into two cell groups (27). The ventrolateral (VL) group was consistently present and was composed of many small, densely staining cells. A dorsomedial (DM) group was only intermittently identifiable and could be distinguished on the basis of position as well as somewhat larger cell size (figure 30.3). Human males had significantly more motoneurons in the VL portion of Onuf's nucleus than did females ( $P < 0.025$ ; figure 30.4). No sex difference was observed in the few DM cells seen. Motoneuron number was not correlated with the age of the subject in either cell population for either sex. No sex difference in estimated nucleus length was observed.

### Discussion

A sex difference was found in the number of motoneurons in Onuf's nucleus of dogs and in VL Onuf's nucleus of humans; early androgen treatment eliminated the dimorphism in dogs. As in humans, Onuf's nucleus of cats is divided into two cell groups. Retrogradely transported fluorescent dye labels cells in VL Onuf's nucleus after injection of the sexually dimorphic IC muscle of cats, while DM cells are labeled after injection of the external anal sphincter (28). Therefore, the human sex difference in motoneuron number



**Figure 30.4**

(A) The number of motoneurons in the VL and DM cell groups of Onuf's nucleus in humans. Males had more motoneurons in the VL nucleus than did females ( $P < 0.025$ ). There was no significant sex difference in the number of DM cells (A) or in the rostrocaudal extent of Onuf's nucleus (nucleus length in  $\mu\text{m}$ ) (B).

was found in that portion of Onuf's nucleus that, in cats, innervates sexually dimorphic muscles. While this study was in progress, a comparable sex difference in the number of Onuf's nucleus motoneurons labeled by applying horseradish peroxidase to the pudendal nerve of macaques was reported (29).

The spinal nucleus of the BC muscle (SNB) of rats is homologous to Onuf's nucleus in that it innervates the BC. Male rats have many more SNB motoneurons than do females (30). Although females are born with the perineal muscles and motoneurons innervating them, both the muscles and motoneurons die shortly after birth. BC muscles and SNB cells can be spared in female rats, however, by perinatal treatment with androgen (3). Evidence suggests that androgen acts on the perineal muscles to enhance motoneuron survival (31, 32).

The sexual dimorphism in the number of perineal motoneurons is less pronounced in dogs and humans than it is in rats (30), as might be expected because female humans and dogs retain perineal muscles (modified in form compared with those of males), while the muscles involute in female rats. Although we cannot be certain how this sex difference in the human spinal cord comes about, the studies of Onuf's nucleus in dogs and the SNB in rats suggest a critical role of perinatal androgen, as early administration of androgen reduces or eliminates the sex difference in motoneuronal number. Furthermore, androgen causes the sex difference in rats by rescuing SNB cells that otherwise would die during the normal period of motoneuronal death (33, 34). We recently have identified a decline in the total number of spinal motoneurons in human fetuses of both sexes (35) that occurs during the time

of androgen production by males (36, 37). The testicular secretion of androgen by human male fetuses may spare some motoneurons in Onuf's nucleus from cell death, resulting in the greater number of neurons in adult males. Interestingly, Onuf's nucleus is particularly resistant to the motoneuronal degeneration characteristic of amyotrophic lateral sclerosis (17). If this resistance is a residuum of steroidal inhibition of cell death during development, then the degeneration of these perineal motoneurons in females may provide a model for the pathology of amyotrophic lateral sclerosis.

We thank Darrell Hall and Karen Rasmussen for technical assistance. We gratefully acknowledge the contribution by Frank Beach and Mike Buehler of the beagles used in this study and the Armed Forces Institute of Pathology for use of the Yakovlev collection and photomicrographs of human material. This work was supported by National Institutes of Health Grant NS19790 and National Science Foundation Grant BNS8451367.

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## Introduction

The brain plays an important part in the initiation and coordination of reproductive functions. These functions are different in the two sexes. In spontaneously ovulating species for example, the female but not the male has a cyclic pattern of secretion of pituitary gonadotrophins which is associated with periodic ovulation and behavioural oestrus. If rats are gonadectomised and primed with oestrogen, the administration of progesterone leads to an increased secretion of luteinising hormone and an enhancement of behavioural receptivity in the female but not in the male (9, 85). By investigating the structural organisation of those parts of the central nervous system thought to be concerned in reproductive functions we have attempted to establish an anatomical basis for such functional dimorphism.

A variety of different experiments have shown that the tuberal hypothalamus is essential for the maintenance of a basal level of secretion of anterior pituitary gonadotrophins, but that the integrity of the preoptic area is necessary for the cyclic surge of gonadotrophins preceding ovulation (29, 45). The preoptic area may also be involved in mating behaviour (59). The characteristic sexual differences in the control of gonadotrophins and certain aspects of mating behaviour depend not on the genetic sex of the animal but upon whether the brain has been exposed to androgen during a critical perinatal period of development (within the first two weeks of life in the rat [43]). This has led to the view that the preoptic area is a specific site for the action of androgen in sexual differentiation (5, 40).

There are several observations which show that in addition to the hypothalamus and preoptic area, other parts of the brain may also have important roles in reproductive function. This applies particularly to the amygdala (for reviews see Raisman and Field [73], Sawyer [79]), and to those of its projection fibres which are distributed through the stria terminalis to the preoptic area and ventromedial nucleus of the tuberal hypothalamus (21, 47, 58).

In a previous ultrastructural study (34) the technique of orthograde degeneration was used to demonstrate

that the fibres of the stria terminalis establish synaptic contacts in the neuropil of the dorsal part of the medial preoptic area and in the relatively cell free region lying between the ventromedial and arcuate nuclei of the hypothalamus. In the neuropil of both regions the synapses of non-strial origin were mainly axodendritic, the majority of axon terminals making contact directly with the dendritic shafts, the remainder contacting dendritic spines. In both males and females the number of spine synapses was consistently higher in the ventromedial nucleus than in the preoptic area, thus providing evidence for a morphological difference between the neuropil of the areas of presumed involvement in the control of the basal as opposed to the cyclic release of gonadotrophins.

A preliminary series of experiments with albino rats of the Wistar strain showed that the neuropil of the preoptic area has more non-strial synaptic contacts on dendritic spines in the female than in the male. No such difference was found in the ventromedial nuclei (74). If such a structural difference is indeed correlated with sexually dimorphic functions such as ovulation, then it too should be determined by the presence or absence of androgens during the first few days after birth in the rat (43). The present account deals with a combined morphological and functional study of a larger series of rats, consisting of normal males, normal females, females treated with androgen either during or after the critical period, and males castrated either within 12 h of birth (before the critical period), or at 7 days of age, or as adults.

The results will be presented in two sections. The first consists of a general account at the light and electron microscopic levels of the anatomical organisation of the preoptic area and the ventromedial nucleus. In particular certain structural characteristics of the dorsal part of the preoptic area (in which the sexually differentiated zone is located) do not appear to have been fully described in previous anatomical studies. The second section is concerned with the quantitative ultrastructural analysis of those parts of the preoptic area and ventromedial nucleus receiving connections from the amygdala through the stria terminalis. In these

regions the incidences of the various types of synapse have been compared in the untreated males and females and in the animals subjected to neonatal hormone manipulations. A preliminary account of some of the anatomical findings has been given in a review by Brown-Grant (9).

### Material and Methods

The 64 animals used in this study were from the litters of 16 rats of a hooded Lister strain (Scientific Products Farm Ltd., Ashford, Kent). The day of birth was taken as day 0; litters were reduced to 6 pups within the first week of life and were weaned on day 21. Males from a series of 7 litters were castrated under hypothermia either within the first 12 h after birth or on day 7. The females of these litters served as the group of untreated females and two litters were left unoperated to serve as control males (some of which were castrated as adults). Female mating behaviour was tested in 5 males castrated within 12 h of birth, 4 males castrated on day 7, and 6 males castrated as adults. These animals are identified by an asterisk in table 31.2. The procedure involves measuring the change in receptivity (lordosis quotient) induced by sequential administration of oestrogen followed by progesterone (for details see Brown-Grant [9]). In a further series of 7 litters, 1.25 mg of testosterone propionate in 0.05 ml of ethyl oleate was administered s.c. to the females either on day 4 or day 16. The males of these litters were not used. In these female rats vaginal smears were taken for a period of 10 consecutive days beginning at approximately 60 days of age, and for a further 10 day period beginning at 90 days of age (to check that a delayed onset of anovulation had not occurred [40]).

Selection of the area for ultrastructural analysis was based on the presence of synapses formed by axons of amygdaloid origin travelling in the stria terminalis. In order to identify these synapses unilateral surgical transection of the stria terminalis was carried out when the rats had reached 80–110 days of age (for technique, see ref. 10). At operation, the mean body weights were  $200 \pm 4$  g (standard error) for the untreated females,  $235 \pm 9$  g for the females androgenised on day 4,  $191 \pm 8$  g for the females androgenised on day 16,  $296 \pm 7$  g for the intact males,  $234 \pm 6$  g for the males castrated on day 0, and  $257 \pm 8$  g for the males castrated on day 7. After a survival period of 2 days the animals were anaesthetised and perfused with a mixture of 1% glutaraldehyde and 1% formaldehyde in 0.1 M phosphate buffer at pH 7.2. At autopsy the genital tracts and ovaries of the females were examined; in the males the completeness of the castration was checked.

The brain was removed and coronal blocks of about 1 mm thick were taken through the preoptic area and ventromedial hypothalamus, treated with 2% buffered osmium tetroxide, dehydrated and embedded in Araldite. Ultrathin sections were cut and stained with lead citrate and uranyl acetate. In each case the remainder of the brain was processed and sectioned for light microscopy (for details see ref. 10) in order to check the completeness of the lesion. Only the animals in which the stria terminalis was completely destroyed were used for the ultrastructural study. Some relevant aspects of the distribution of degeneration after such a lesion have been described (34). Apart from the fimbria and the stria terminalis, the lesion does not damage any other structures known to send fibres to the particular parts of the preoptic area and ventromedial nuclei sampled in this study.

The present quantitative ultrastructural analysis is based on a “4-way” classification of synapses—i.e. into shaft (figures 31.11, 31.12, 31.15) versus spine (figures 31.11, 31.13, 31.14, 31.16, 31.17) synapses and degenerating (figures 31.14–31.17) versus non-degenerating (figures 31.11–31.13) synapses. In a small proportion of synapses it was not possible to determine with certainty whether the postsynaptic element was a shaft or a spine. These synapses were recorded as of unknown termination; their number was never large enough to affect the quantitative analysis.

The stria terminalis was completely sectioned on the left side two days prior to sacrifice in 64 animals. These animals consist of 6 groups: 16 untreated adult females (F), 14 adult females treated with 1.25 mg of testosterone propionate on the 4th day of postnatal life (F4), 7 adult females treated with 1.25 mg of testosterone propionate on the 16th day of life (F16), 11 adult males (M)—5 of which were intact and 6 castrated as adults—9 adult males castrated within 12 h of birth (M0), and 7 males castrated on the 7th day of life (M7). For convenience in description, groups F, F16 and M0 will be referred to as “cyclic” (i.e. their presumed pattern of gonadotrophin release) and groups M, M7 and F4 as “non-cyclic.” Ultrathin sections were taken from the preoptic area of every animal (figure 31.3) and from the ventromedial nucleus (figure 31.4) of 3 animals from each of the 6 groups. For each of the two areas the sections were identified by a code number and the original identity of any animal was not known until all the counts from all animals had been completed. The figures for the preoptic area consisted of counts of all grid squares that contained more than one degenerating terminal, excluding the row of grid squares (i.e. about  $70 \mu\text{m}$ ) immediately below the anterior commissure (arrows on figure 31.19) since this row contains occasional spiny dendrites from cells in the

bed nucleus of the stria terminalis. A total of 50,773 synapses were counted in 869 grid squares from the 64 animals. This consisted for each animal of a mean count of 800 synapses (range 400–1700) in a mean of 14 grid squares (range 8–26). All the grid squares immediately surrounding the area containing degeneration were also scanned so as to exclude the presence of degenerating terminals. The samples from the ventromedial nucleus consisted of 10 grid squares taken from the cell-poor region between the ventromedial and arcuate nuclei and containing degenerating terminals. A total of 11,701 synapses were counted in the 18 animals. This consisted for each animal of a mean count of 650 synapses (range 570–740). In neither area were any other criteria used either to select or to reject grid squares.

For the purposes of presentation, the incidences of the 4 types of synapses are expressed as the number of synapses per grid square; this comprises an area of about 1760 sq.  $\mu\text{m}$ . It is convenient to express the degree of scatter in the data by using the standard error. In the case of both the non-strial shaft and spine synapses in the ventromedial nucleus, the incidence per grid square is greater than 12 and it seems justified to employ the *t* test to assess the significance of observed differences. (The assumption of a roughly normal distribution was also supported by a goodness of fit test.) However, in the case of both types of strial synapses in the ventromedial nucleus and all the 4 types of synapse in the preoptic area, there are fewer synapses per grid square, and to avoid the assumptions required for parametrical statistical tests we have ranked the data and used a preliminary analysis of variance by the Kruskal–Wallis test, followed by individual comparisons between groups by the Mann–Whitney U test (82). Because of the large number of different animals it was sufficient to base the comparisons for the preoptic area on the pooled figures for all the grid squares for each animal. In the case of the ventromedial nuclei, it was originally planned to use the individual values for each of the 180 grid squares. However, since there turned out to be a large proportion of ties (which make the Mann–Whitney U test cumbersome), the values for the individual grid squares were grouped at random into sets of 5 (each group including one or two squares from each of the 3 animals within that treatment group).

A further series of animals was prepared for light microscopic investigations. After section of the stria terminalis, the pattern of orthograde degeneration was examined by a modification (96) of the Fink–Heimer method or by the method of De Olmos and Ingram (22). Other histological techniques included a modification of the rapid Golgi method (94) and the fluorescence technique for biogenic amines (8, 19, 50).

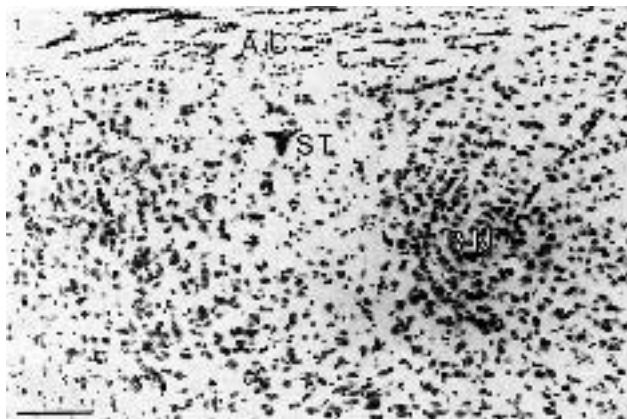
## Results

### General Anatomical Features of the Preoptic Area

The fibres of the stria terminalis can be traced from the position where they form a compact bundle located at the dorsolateral margin of the thalamus, forward and ventrally to reach the anterior commissure, to which they are closely applied. Passing over the rostral and caudal aspects of the commissure, they turn backwards into the preoptic area. At successive caudal levels the fibres become gradually more ventrally placed and enter the anterior hypothalamus. In the absence of any other nomenclature (e.g., refs. 14, 41) the part of the preoptic area traversed by the stria terminalis will be designated the *strial part of the preoptic area*. It is somewhat triangular in section, lying just beneath the anterior commissure, between 0.5 and 1.0 mm from the midline at the level of the most rostral part of the third ventricle (figure 31.3). This corresponds to a small mid-dorsal part of the medial preoptic area. This triangular area is bounded on its dorsal aspect by the anterior commissure, and on its ventral, medial and lateral aspects by parts of the preoptic area not containing strial fibres. In the quantitative ultrastructural study samples were taken only as far rostrally as the level of crossing of the anterior commissure, and caudally samples were discontinued at the level where the fibre bundles of the stria terminalis are no longer in contact with the anterior commissure. The area thus defined has a maximum diameter of about 300  $\mu\text{m}$  in all directions; it can be readily located and its position is consistent from one animal to another. It is located at approximately A 7.3, L 0.7, H –0.6 in the atlas of De Groot (20).

In a series of sections stained with thionin this triangular area is seen to be relatively devoid of neurones, probably because a major part is taken up by the massive bundles of myelinated strial axons. Medially and ventrally it is bounded by zones of fairly small neurones, while on its lateral aspect is a rounded compact mass of cells. The position of this distinct nucleus is indicated by the letters RN (“round nucleus”) in figures 31.1 and 31.9. Serial reconstruction shows that it is roughly spherical, about 300  $\mu\text{m}$  in diameter, and is contiguous on its lateral aspect with the most ventral part of the bed nucleus of the stria terminalis which caps the dorsal, lateral and ventral aspects of the lateral margin of the anterior commissure at this level (figure 31.3). Caudally the round nucleus merges into a less well defined region.

Reference to sections stained to show orthograde degeneration 2–5 days after a lesion of the stria terminalis shows a characteristic distribution of degeneration. The triangular area containing the strial fibres is densely packed with argyrophilic fragments, the larger

**Figure 31.1**

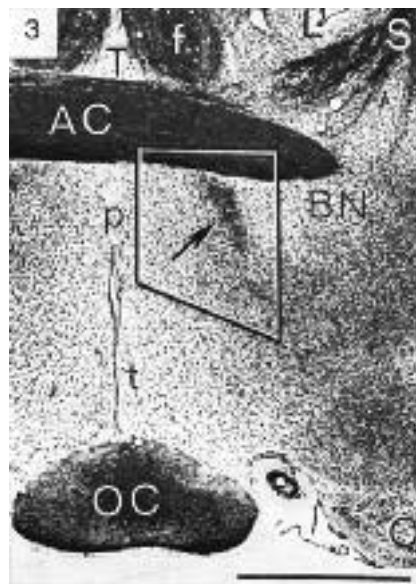
A light photomicrograph of a coronal section through the strial part of the preoptic area, showing the position of the “round nucleus” (RN) lateral to the strial bundle (ST). AC, anterior commissure. Nissl stain. Scale bar, 100  $\mu$ m.

**Figure 31.2**

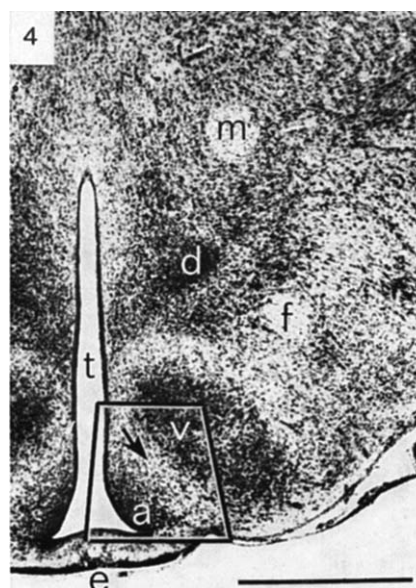
A light micrograph of a coronal section through the strial part of the preoptic area (ST) showing the distribution of degeneration after a lesion of the stria terminalis. AC, anterior commissure; BN, bed nucleus of the stria terminalis; RN, round nucleus (which is free of degeneration). Wiitanen modification of the Fink–Heimer stain. Scale bar, 100  $\mu$ m.

part of which are due to the degenerating myelinated fibres, although electron microscopy reveals the additional presence of degenerating terminals (see below). The “round nucleus” on the lateral aspect of this area is completely free of degeneration (figure 31.2), and lateral to this the bed nucleus of the stria terminalis is filled with a dense, rather evenly spaced mass of argyrophilic fragments strongly suggestive of terminal degeneration (an interpretation confirmed at the ultrastructural level).

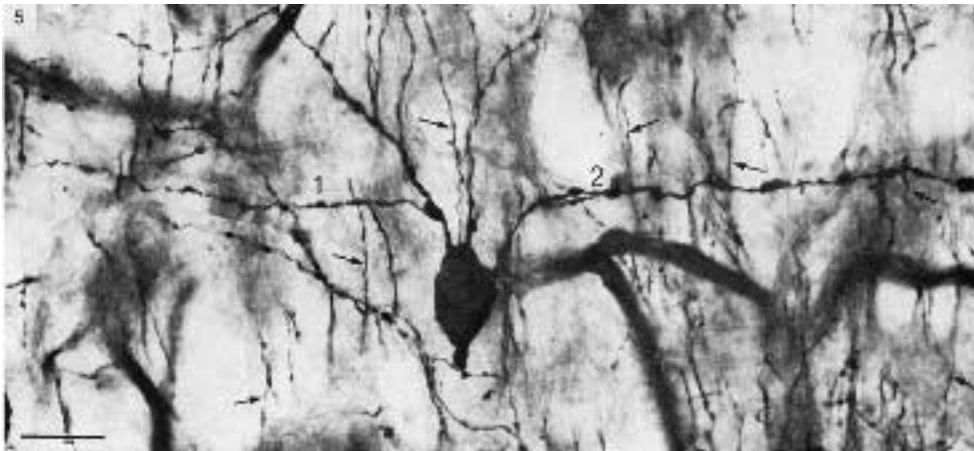
In sections stained by the rapid Golgi method (94) the axons of the stria terminalis can be seen coursing vertically downwards, and the cells of the region through which they pass tend to have dendrites lying

**Figure 31.3**

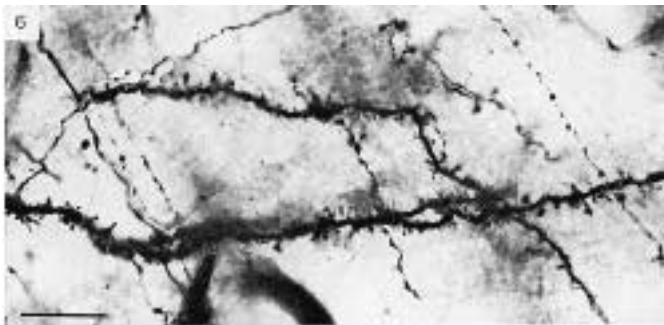
A light photomicrograph of a coronal section through the preoptic area. The box shows the location of the area taken for ultrathin sections. Arrow, strial part of the preoptic area. AC, anterior commissure; BN, bed nucleus of the stria terminalis; f, fornix; L, ventral tip of the lateral ventricle; O, olfactory tubercle; OC, optic chiasma; p, periventricular part of the preoptic area; S, main part of the stria terminalis; t, third ventricle; T, triangular septal nucleus (note the commissural component of the stria terminalis just ventral to this, on the dorsal aspect of the anterior commissure). Bodian stain. Scale bar, 1 mm.

**Figure 31.4**

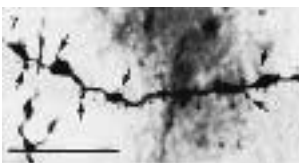
A light photomicrograph of a coronal section through the medio-basal part of the tuberal hypothalamus showing the cell free zone (arrow) lying between the arcuate nucleus (a) and the ventromedial nucleus (v). The box outlines the region sampled for electron microscopy. d, dorsomedial nucleus; e, median eminence; f, fornix; m, mammillothalamic tract; t, third ventricle. Nissl stain. Scale bar, 1 mm.

**Figure 31.5**

A cell in the strial part of the preoptic area with beaded dendrites arising from its dorsal pole and running medially (1) and laterally (2) at right angles to the axons of the stria terminalis (some of which are shown by arrows) which run dorsoventrally through the field. Rapid Golgi method. Scale bar, 20  $\mu$ m.

**Figure 31.6**

Parts of two non-varicose, spine bearing dendrites from the bed nucleus of the stria terminalis. Rapid Golgi method. Scale bar, 20  $\mu$ m.

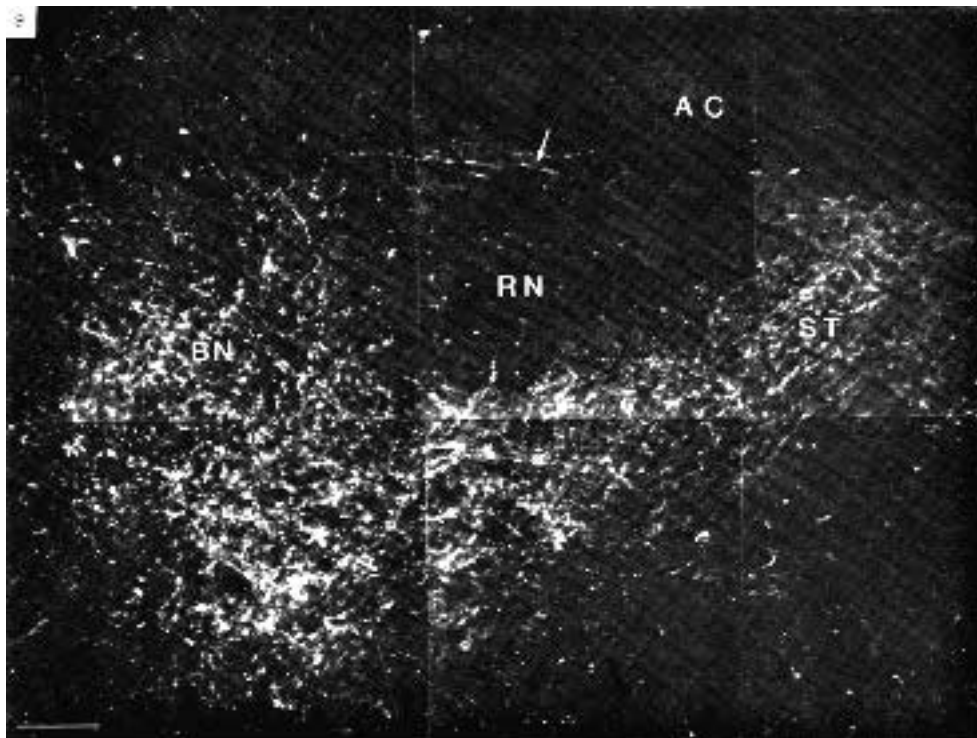
**Figure 31.7**

Part of a beaded dendrite from the bed nucleus of the stria terminalis. Arrows, spines. Rapid Golgi method. Scale bar, 20  $\mu$ m.

**Figure 31.8**

A fluorescence photomicrograph of part of an axon entering the strial part of the preoptic area. Scale bar, 20  $\mu$ m.

in the horizontal plane and directed medially and laterally from the cell bodies so as to intersect in a rectilinear lattice with the strial axons (figure 31.5). These dendrites are varicose and bear only a few spines (figures 31.5, 31.7). The rectilinear lattice has been clearly seen in 6 brains, and the fragmentary observations possible in a large number of other brains are consistent with this interpretation. These observations have been confirmed at the electron microscopic level, where varicose spine free dendritic shafts (figure 31.10) are seen predominantly orientated in the horizontal plane. The cells of the bed nucleus of the stria terminalis present a very different appearance in Golgi material (see also Valverde [94]). The dendritic shafts are more branched, the branches insinuating themselves in all directions through the nucleus. In contrast to the cells of the strial part of the preoptic area, the dendrites in the bed nucleus have many spines (figure 31.6). Several such spiny dendrites can be seen closely applied to the anterior commissure and also winding around to its ventral surface. Because of this arrangement, occasional spiny



**Figure 31.9**

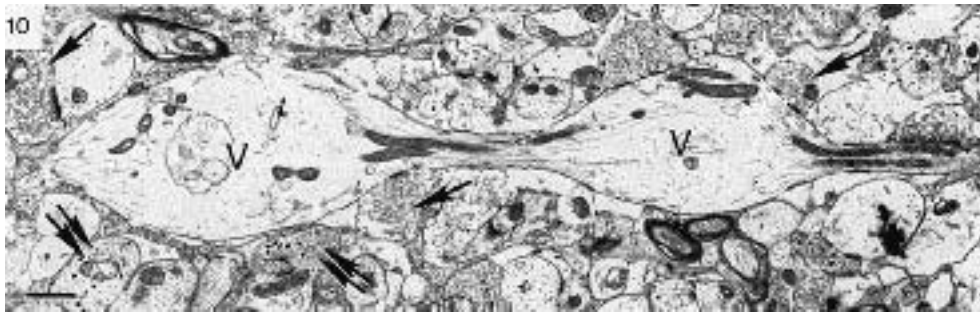
A montage of several light micrographs showing the fluorescent fibre plexus extending from the ventral part of the bed nucleus of the stria terminalis (BN) into the strial part of the preoptic area (ST) and leaving the round nucleus (RN) relatively free of fluorescent fibres. AC, anterior commissure; arrow, fluorescent fibre passing dorsal to the round nucleus. Scale bar, 100  $\mu$ m.

dendrites penetrate into the strial part of the preoptic area, especially into its most dorsal layer immediately below the anterior commissure. For this reason this layer (marked by arrows in figure 31.18) was excluded from the ultrastructural samples.

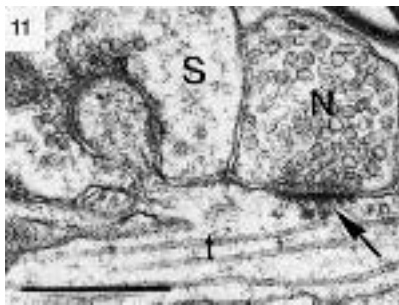
Fuxe (36) has shown that a dense plexus of green, brightly fluorescent fibres is present in the bed nucleus of the stria terminalis. In our own material, sectioned on the Vibratome (Oxford Instruments, California, U.S.A. [50]) we have been able to confirm this observation and also to show that whereas laterally this fluorescent plexus is restricted within the confines of the bed nucleus, medially it extends to a point more than half way to the third ventricle, tapering as it does so. The most medial fibres (figure 31.8) curve dorsally so as to reach the ventral border of the anterior commissure, and in doing so they leave a zone just below the lateral part of the anterior commissure which is relatively free of fluorescent fibres (figure 31.9). Rostrally this free zone becomes narrower, whereas caudally it becomes progressively more extensive. Thionin staining of the sections previously examined and photographed for fluorescence showed that the fluorescence free zone is occupied by the rounded mass of cells ("round nucleus," figure 31.1) which is free of degeneration after lesions of the stria terminalis, and that the

tapering medial extension of fluorescent fibres enters the strial part of the preoptic area from its ventral border (figure 31.9). Apart from the rather weaker plexus of fluorescent fibres in the periventricular zone, the remainder of the dorsal preoptic area is devoid of fluorescence.

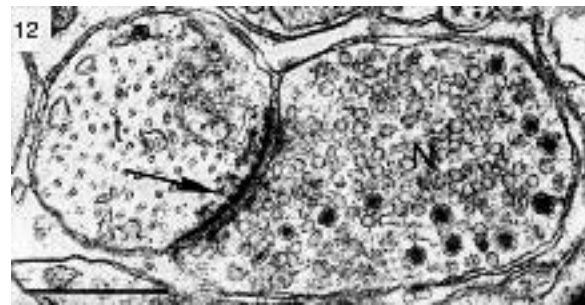
**Electron Microscopy of the Preoptic Area** The sections taken for electron microscopy were about 1 mm  $\times$  1 mm in size and included the ventral edge of the anterior commissure, the whole of the strial part of the preoptic area, and a substantial margin of tissue on its medial, lateral and ventral aspects (figure 31.3). This region contains small or medium sized neuronal cell bodies whose dendrites are often varicose (figure 31.10) and are orientated in a predominantly mediolateral direction. Such dendrites have very few spines, and those which are present tend to be narrow, with highly dense cytoplasm (figures 31.11, 31.13, 31.14). These spines almost always contain a number of ribosome like particles, but they have not been seen to contain a spine apparatus. The neck is commonly very short, although long narrow necks have occasionally been found (figure 31.14). Dendrites bearing many spines are seen towards the lateral edge of the sections. In these cases the spines are much larger, having narrow

**Figure 31.10**

An electron micrograph of a beaded dendrite from the strial part of the preoptic area. Mitochondria and microtubules are concentrated in the narrow segments connecting the varicosities (V). Single arrows, nerve terminals with few dense core vesicles; double arrows, nerve terminals with many dense core vesicles. Compare with the Golgi stained material (figure 31.5). Scale bar, 1  $\mu$ m.

**Figure 31.11**

A dendritic shaft containing microtubules (t) is contacted directly by an axon terminal (N) making a "shaft synapse" and bears a small dark spine which is contacted by a second axon terminal (S) making a "spine synapse." Arrow, asymmetrical thickening with subjunctional bodies. Scale bar, 0.5  $\mu$ m.

**Figure 31.12**

A dendritic shaft containing microtubules (t) is contacted by an axon terminal (N) containing synaptic vesicles and large numbers of dense core vesicles. Arrow, asymmetrical synaptic thickening with a row of subjunctional bodies. Scale bar, 0.5  $\mu$ m.

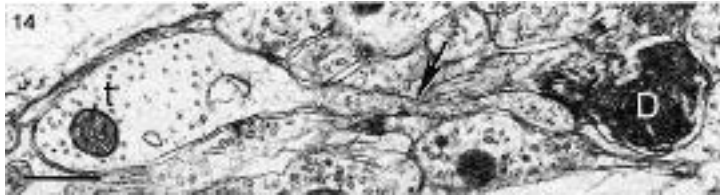
necks and wide terminal expansions which may contain quite elaborate spine apparatus (figures 31.16, 31.17). Such dendrites and spines are also seen occasionally in the layer immediately adjacent to the anterior commissure and comparison with the Golgi stained material suggests that these spiny dendrites belong to cells lying in the bed nucleus of the stria terminalis, an interpretation which is supported by the observation of similar spiny dendrites in electron microscopic sections taken from the main part of the bed nucleus.

The strial part of the preoptic area contains prominent bundles of myelinated axons, a large proportion of which show electron dense degeneration two days after a lesion of the stria. The majority (88%) of all axon terminals terminate on dendritic shafts, with about 7% on dendritic spines and 4–5% on cell bodies. Occasionally two axon terminals contact the same dendritic spine. Most axon terminals contain clear synaptic vesicles of about 45 nm diameter, with occasional dense core vesicles of up to 100 nm diameter or larger. Some terminals and preterminals contain much higher proportions of dense core vesicles (figures 31.10,

31.12). Most common are spherical dense core vesicles of about 70 nm diameter mixed with spherical synaptic vesicles of 45 nm diameter. At times, however, the dense core vesicles are more irregularly shaped, the synaptic vesicles are flattened, and the profile may contain irregular flattened sacs. The incidence of flattened synaptic vesicles cannot be readily assessed using the present method of fixation (92). Clear distinction between symmetrical and asymmetrical types of synaptic thickening (18) can be made in a large proportion of synapses; in both the preoptic area and the ventromedial nucleus the axosomatic synapses are mainly of the symmetrical type, the synapses on dendritic shafts are of either type, and the synapses on dendritic spines almost all have asymmetrical synaptic thickenings. This applies to both the strial (degenerating) and the non-strial synapses on spines. The degenerating axon terminals are readily identified by their reaction of increased electron density and collapse at two days after section of the stria terminalis (figures 31.14–31.17). Approximately 4–5% of the terminals undergo degeneration and these terminals are distributed approximately equally on dendritic shafts and spines (see below).

**Figure 31.13**

A dendritic shaft containing microtubules (t) bears a small dark spine which is contacted at an asymmetrical thickening (arrow) by an axon terminal (N). Scale bar, 0.5  $\mu\text{m}$ .

**Figure 31.14**

A dendritic shaft containing microtubules (t) bears a small dark spine on a long slender neck (arrow). The spine is contacted at an asymmetrical synaptic thickening by a degenerating ("strial") axon terminal showing the electron dense reaction of orthograde degeneration (D). Scale bar, 0.5  $\mu\text{m}$ .

**Figure 31.15**

A dendritic shaft containing microtubules (t) is contacted at an asymmetrical synaptic thickening (arrow) by a degenerating axon terminal (D) in which the outlines of synaptic vesicles can still be recognised ("strial shaft synapse"). Scale bar, 0.5  $\mu\text{m}$ .

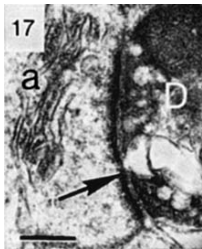
In order to correlate the ultrastructural observations with the light microscopical information, "maps" based on a classification of axon terminals and other structures were constructed for the strial part of the preoptic area and the surrounding regions (figure 31.18). For this purpose axon terminals were divided according to their mode of termination on cell bodies, dendritic shafts or dendritic spines, and further subdivided into 3 groups according to the number of dense core vesicles present. Degenerating myelinated axons, non-myelinated fragments and terminals were also recorded. Based on counts of 20,644 structures from 254 grid squares, two such maps were reconstructed,

one for a normal adult male, and one for a female. These maps illustrate several features:

1. The myelinated axons of the stria terminalis form a compact bundle lying against the anterior commissure and about 200–300  $\mu\text{m}$  in diameter.
2. This bundle is surrounded by a more extensive zone containing degenerating axon terminals involved in synaptic contacts. This zone extends for 300–400  $\mu\text{m}$  below the anterior commissure, beginning about 100–200  $\mu\text{m}$  medial to the strial bundle, and extending to at least 400–500  $\mu\text{m}$  lateral to it. It is bounded on all sides by regions of neuropil not containing degenerating terminals.
3. A region could be defined by the presence of large proportions of terminals and preterminals containing 5 or more dense core vesicles. This region forms a band overlapping the ventral and lateral aspects of the area containing degenerating terminals. The axon terminals with dense core vesicles may belong to the fluorescent fibres described above (see refs. 37, 49, 72) but the electron microscopic maps cover too small an area to allow adequate correlation with the overall distribution of fluorescent fibres in the preoptic area.
4. A further region was defined as the area containing more than 10% of synapses on dendritic spines. The extent of this region was different in the two sexes. *In the female* its boundaries corresponded medially and ventrally with those of the area containing degenerating synapses, although it continued further laterally, probably to join up with the bed nucleus of the stria terminalis. *In the male* only small patches of neuropil had more than 10% of synapses on dendritic spines. These

**Figure 31.16**

A dendritic shaft containing microtubules (t) bears a fairly large spine on a narrow neck (double arrow). The spine contains a small area of spine apparatus (a) and is contacted at an asymmetrical synaptic thickening (arrow) by a degenerating axon terminal (D) in which may be seen the remnants of mitochondria and synaptic vesicles ("strial spine synapse"). Scale bar, 0.5  $\mu\text{m}$ .

**Figure 31.17**

The junctional region between a degenerating axon terminal (D) and a dendritic spine ("strial spine synapse") containing a well developed spine apparatus (a). Arrow, asymmetrical synaptic thickening. Scale bar, 0.2  $\mu\text{m}$ .

areas tended to lie up against the anterior commissure and in the lateral part of the section—i.e., in regions in which the Golgi stained material shows spiny dendrites arising from cell bodies lying in the bed nucleus.

#### General Anatomical Features of the Ventromedial Nucleus: Light and Electron Microscopy

Several studies (e.g., refs. 41, 47, 84) have described the ventromedial hypothalamic nucleus. Its cells form a compact mass, which is surrounded by a relatively cell free zone (figure 31.4) containing the peripheral parts of the dendrites. Medioventrally this zone is about 150  $\mu\text{m}$  wide and immediately adjoins the arcuate nucleus. The fibres of the stria terminalis form synapses in the peripheral shell of the ventromedial nucleus (47) and probably also (but to a lesser extent) in its central parts (21). Samples for the ultrastructural study were taken from the relatively cell free region lying between the ventromedial nucleus and the arcuate nucleus at the level of the mid-tuberal hypothalamus (figure 31.4). In a recent study of the location of cells which could be activated antidromically by electrical stimulation of the median eminence Makara et al. (63) have shown that cells in the ventromedial region of the ventromedial nucleus as well as those in the adjacent arcuate nucleus (84) are the direct source of axons running to the median eminence. It is this ventromedial part of

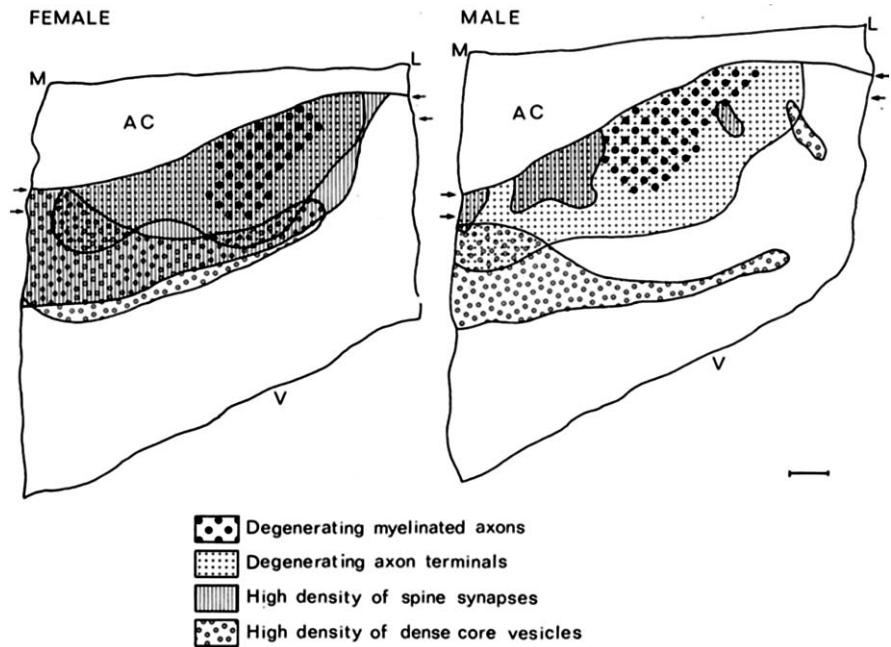
the ventromedial nucleus which is included by Halász (42) in his hypothalamic "islands."

Some ultrastructural features of the ventromedial nucleus have been mentioned in a previous report (34). The neuropil of this area is basically similar to that of the preoptic area. The spine bearing dendrites of the ventromedial nucleus, which are seen in the Golgi stained sections, can also be identified at the electron microscopic level, but there are also present varicose, spine-poor dendrites of the type found in the preoptic area. The general features of synapses in the ventromedial nucleus are similar to those in the preoptic area but with a higher proportion of terminals making contact with dendritic spines. The synapses are distributed roughly 75% on dendritic shafts, 22% on dendritic spines, and 3–4% on cell bodies. As well as the small dark spines of the type found in the preoptic area, the ventromedial nucleus also contains spines of the large type, containing a spine apparatus. Axon terminals and preterminals containing high proportions of dense core vesicles were less common in the ventromedial nucleus than in the preoptic area. Two days after section of the stria terminalis approximately 13% of the axon terminals show the electron dense reaction of ortho-grade degeneration.

#### Sexual Dimorphism: Quantitative Ultrastructural Analysis of the Neuropil of the Preoptic Area and Ventromedial Nucleus

Table 31.1 shows the incidences of the different types of synapse in both the preoptic areas and the ventromedial nuclei of the 6 different groups of animals. The main features of the distribution are as follows:

1. In both areas and in all 6 groups the non-strial shaft synapses outnumber the non-strial spine synapses. This preponderance of shaft over spine synapses is about 12:1 for the preoptic area but only 3:1 for the ventromedial nucleus.
2. In the case of the terminals of strial origin, synapses on dendritic shafts are about as common as those on spines in the preoptic area but in the ventromedial



**Figure 31.18**  
Reconstructions of ultrathin sections through the strial part of the preoptic area from one female rat and one male rat. The symbols show the location of the degenerating myelinated axons of the stria terminalis bundle (large dots), the degenerating axon terminals (small dots), the areas having more than 10% of synapses on dendritic spines (vertical hatching), and the areas with high proportions of dense core vesicles (open circles). On the dorsal border of the section the row of grid squares immediately below the anterior commissure (AC) is marked by arrows and was excluded from the quantitative study (see text). L, lateral; M, medial; V, ventral. Scale bar, 100  $\mu$ m.

**Table 31.1**  
The incidences per grid square (mean  $\pm$  standard error) of the 4 types of synapse in the preoptic area (POA) and ventromedial nucleus (VMH) in the 6 groups of animals

		Endocrine Status					
		Cyclic			Non-Cyclic		
		F (16)	F16 (7)	M0 (9)	M (11)	M7 (7)	F4 (14)
<i>POA</i>							
Non-strial	shaft	50.0 $\pm$ 2.0	53.1 $\pm$ 3.0	54.3 $\pm$ 3.0	55.2 $\pm$ 1.4	52.6 $\pm$ 1.3	48.4 $\pm$ 2.4
	spine	5.3 $\pm$ 0.3	5.4 $\pm$ 0.5	5.0 $\pm$ 0.3	3.3 $\pm$ 0.2	3.9 $\pm$ 0.4	3.5 $\pm$ 0.2
Strial	shaft	1.2 $\pm$ 0.1	1.0 $\pm$ 0.1	1.2 $\pm$ 0.1	1.2 $\pm$ 0.1	0.7 $\pm$ 0.1	0.9 $\pm$ 0.1
	spine	1.6 $\pm$ 0.2	1.4 $\pm$ 0.3	1.8 $\pm$ 0.2	1.7 $\pm$ 0.1	1.3 $\pm$ 0.1	1.6 $\pm$ 0.2
<i>VMH</i>							
Non-strial	shaft	46.3 $\pm$ 1.8	44.4 $\pm$ 1.6	43.6 $\pm$ 1.6	42.4 $\pm$ 1.5	42.3 $\pm$ 1.4	42.8 $\pm$ 1.8
	spine	12.9 $\pm$ 0.7	12.6 $\pm$ 0.8	13.2 $\pm$ 0.9	12.0 $\pm$ 0.7	13.9 $\pm$ 0.9	13.1 $\pm$ 0.9
Strial	shaft	2.3 $\pm$ 0.2	1.6 $\pm$ 0.2	1.9 $\pm$ 0.3	1.9 $\pm$ 0.3	1.8 $\pm$ 0.2	1.9 $\pm$ 0.2
	spine	6.2 $\pm$ 0.5	6.2 $\pm$ 0.6	6.6 $\pm$ 0.5	7.0 $\pm$ 0.6	6.7 $\pm$ 0.4	6.5 $\pm$ 0.5

The “cyclic” group consists of *F* (normal females), *F16* (females treated with androgen on day 16), and *M0* (males castrated within 12 h of birth); the non-cyclic group consists of *M* (normal males), *M7* (males castrated on day 7) and *F4* (females androgenised on day 4). Number of rats is given in parentheses.

**Table 31.2**

The mean numbers of the non-strial spine synapses per grid square in the preoptic area in each individual animal

	Endocrine Status					
	Cyclic			Non-Cyclic		
	F	F16	M0	M	M7	F4
M94*				2.0		
F103						2.3
M74*					2.6	
M75				2.6		
F110						2.7
M98*				2.7		
F111						2.9
F112						2.9
F101						3.0
F102						3.1
F104						3.1
M82*					3.1	
M95*				3.2		
M99*				3.2		
M79*					3.4	
F97						3.5
M62					3.5	
M76				3.5		
F96						3.6
M96*				3.6		
M71				3.6		
M72				4.0		
M97*				4.0		
M73*			4.0			
F98						4.1
M61				4.1		
F77	4.1					
M60			4.2			
F95						4.2
F109						4.3
F86	4.3					
F80	4.4					
M81*			4.4			
F75	4.4					
F78*			4.5			
F99						4.5
F76	4.5					
F92	4.5					
F72	4.6					
F117		4.6				
M69					4.7	
F114		4.7				
M83*					4.7	
F94	4.7					
F116**		4.9				
F91	4.9					
F90		4.9				
F83	5.0					

**Table 31.2**

(continued)

	Endocrine Status					
	Cyclic			Non-Cyclic		
	F	F16	M0	M	M7	F4
M80*			5.0			
M70			5.1			
F107						5.2
F85	5.2					
F82		5.2				
M63			5.2			
M65					5.2	
F115		5.4				
F73	6.0					
M84*			6.1			
F74	6.2					
F84	6.4					
F93	6.5					
M77			6.5			
F89		8.0				
F69	8.3					

The numbers are ranked in increasing order (from above downwards) to show the lower incidence in the non-cyclic group as compared to the cyclic group (*cf.* table 31.1).

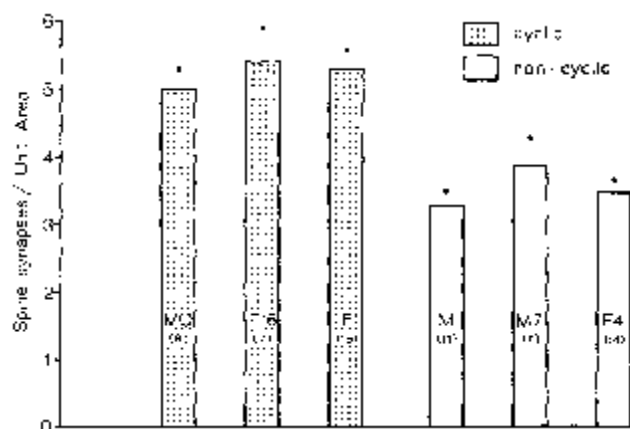
\* Animals subjected to gonadectomy, oestrogen and progesterone treatment and behavioural testing.

\*\* Female treated with androgen on day 16 and showing persistent oestrus.

nucleus they are outnumbered by spine synapses (in the ratio of 1:3).

3. In both areas the strial synapses account for a large proportion (about one-third) of the spine synapses, but only a small proportion (1–2%) of the shaft synapses.

4. Only one category of synapses shows sexual dimorphism. This is the group of non-strial spine synapses in the preoptic area. In the 3 cyclic groups a total of 2230 non-strial spine synapses were found in a total area of  $7.62 \times 10^5$  sq.  $\mu\text{m}$  (432 grid squares). In the non-cyclic groups there were 1555 non-strial spine synapses in  $7.71 \times 10^5$  sq.  $\mu\text{m}$  (437 grid squares). The incidence of these synapses per grid square is  $5.3 \pm 0.3$  in the female and  $3.3 \pm 0.2$  in the male. The other two cyclic groups (*i.e.* the females treated with androgen on day 16 and the males castrated on day 0) have incidences in the female range, thus confirming that by the 16th day of life the female is refractory to the masculinising effects of exogenous androgen, while castration of the male within 12 h of birth is early enough to avoid the masculinising effects of its own (endogenous) androgen. While the untreated male is clearly different from all the 3 cyclic groups (compare column M with columns F, F16 and M0 in table 31.2) the other two non-cyclic groups (*i.e.*, the males castrated on day 7 and the



**Figure 31.19**

A bar diagram showing the mean incidences (dot, one standard error) of non-strial synapses per grid square in each of the 6 groups of animals (cyclic groups shaded). The number in brackets is the number of animals in each group.

females androgenised on day 4) are more variable (figure 31.19). This is due to the fact that 3 out of the 7 males castrated on day 7, and 4 of the 14 females treated with androgen on day 4 have spine synapse incidences which are in the female range. This suggests that the neonatal testis has not had time within the first week of life to secrete enough androgen to masculinise more than a proportion of the animals. Similarly, although all the females treated with androgen on day 4 were anovulatory as adults (see below), this dose and time of administration only results in “masculinisation” of the spine synapse pattern in the preoptic area of about two-thirds of the females. The significance of these differences has been assessed by ranking tests; the ranked values for the individual animals are presented in table 31.2.

The incidences of the remaining 3 types of synapse in the preoptic area and all 4 types in the ventromedial nucleus have also been compared in the 6 cyclic and non-cyclic groups. The results of the statistical analysis of the incidences of all the various categories of synapse are shown in table 31.3 for the preoptic area and table 31.4 for the ventromedial nucleus. Two sets of comparisons were made: in the first each of the 6 groups was compared with each of the other groups (15 comparisons); in the second the 3 non-cyclic groups were pooled and compared with the 3 cyclic groups. Both types of comparison are shown in table 31.3 for the preoptic area. The difference in the incidence of the non-strial spine synapses is highly significant for most of the individual comparisons between individual cyclic and non-cyclic groups and also for the comparison between the pooled cyclic vs. non-cyclic groups (for the larger numbers the significance of  $U$  is assessed

by computation of  $z$  [82]). None of the comparisons between individual cyclic groups or between individual non-cyclic groups was significant. With one exception (which would be expected to arise at random when 30 tests are made) none of the comparisons are significant for the non-strial shaft synapses or for the strial spine synapses. In the case of the strial shaft synapses, there is considerable variability in all comparisons, whether between cyclic and non-cyclic groups or between animals from the same cyclic or non-cyclic group. For this category of synapses, however, the pooled comparisons give a value of  $z$  (2.27) which is hardly significant ( $P = 0.023$ ). This suggests that the variability is of a random nature, of no biological significance in the comparison of the treatment groups. Reference to the original data shows that the number of strial shaft synapses per grid square is very low in the preoptic area, and this is probably the underlying difficulty causing most of the variability.

For the ventromedial nucleus, the statistical significance of the comparisons between the pooled cyclic and non-cyclic groups are shown in table 31.4. Of the 15 individual comparisons for each of the 4 groups (not shown in the tables) 56 fail to reach even the 0.1 level of significance. Of the remaining 4, 3 have a  $P$  value between 0.1 and 0.05 and one (the comparison of the untreated females with the females androgenised on day 16) has a  $P$  value between 0.01 and 0.001. These 4 different comparisons are all in the strial shaft synapses, once again the category of synapses whose incidence is lowest and therefore subject to the greatest inherent variability.

Although not shown in the tables, it was found that neither the total numbers of synapses per grid square nor the percentage of synapses undergoing degeneration were different in the two sexes in either the preoptic area or the ventromedial nucleus.

### Endocrine Observations

The untreated normal adult females showed regular 4 or 5 day vaginal cycles. At autopsy the ovaries were of normal size with normal corpora lutea. All the adult females which had been treated with 1.25 mg of testosterone propionate on the 4th day of life showed persistent vaginal cornification (at least 70% cornified smears) when smeared for two 10 day periods at 60 and 90 days of age. At autopsy the ovaries were small, white and polyfollicular, with no luteal tissue. Conversely, in 6 of the 7 adult females which had been treated with testosterone on the 16th day of life, the vaginal smear pattern and the ovaries at autopsy were indistinguishable from those of the untreated females. The one exceptional rat in this group (F 116) had vaginal smears and ovaries similar to those of the females

**Table 31.3**

The results of a statistical analysis by the Mann–Whitney U-test (calculation of U or z) of the significance (probability, *P*) of the differences in incidences of the 4 types of synapse in the preoptic area

Comparison	Non-Strial				Strial			
	Shaft		Spine		Shaft		Spine	
	U	P	U	P	U	P	U	P
F vs. M	41	++	0	+++	84	ns	66	ns
F vs. M7	36	ns	25	+	18	++	40	ns
F vs. F4	110	ns	16	+++	61	+	104	ns
F16 vs. M	28	ns	0	+++	28	ns	25	ns
F16 vs. M7	19	ns	7	?	11	+	22	ns
F16 vs. F4	30	ns	3	+++	35	ns	40	ns
M0 vs. M	43	ns	1	+++	47	ns	45	ns
M0 vs. M7	20	ns	15	?	3	++	12	?
M0 vs. F4	36	?	15	+++	27	+	53	ns
F vs. F16	35	ns	36	ns	43	ns	45	ns
F vs. M0	39	?	66	ns	64	ns	46	ns
F16 vs. M0	21	ns	20	ns	14	?	21	ns
M vs. M7	23	ns	30	ns	11	++	16	?
M vs. F4	44	?	71	ns	38	+	68	ns
M7 vs. F4	37	ns	35	ns	35	ns	35	ns
	<i>z</i>	<i>P</i>	<i>z</i>	<i>P</i>	<i>z</i>	<i>P</i>	<i>z</i>	<i>P</i>
Cyclic vs. non-cyclic	0.17	0.86	5.76	*	2.27	0.02	0.00	1.00

Top panel shows comparisons between the individual pairs of cyclic (*cf.* Table 31.1) and non-cyclic groups; middle panel, comparisons between individual cyclic or individual non-cyclic groups; lower panel, the comparison of the pooled cyclic with the pooled non-cyclic groups. Significance levels (two-tailed U test); +++, *P* < 0.002; ++, *P* < 0.02; +, *P* < 0.05; ?, *P* < 0.1; ns, *P* > 0.1.

\* *P* < 0.00003.

**Table 31.4**

The results of a statistical analysis by the Mann–Whitney U test (calculation of U, sample sizes *n*<sub>1</sub>*n*<sub>2</sub>) or the *t* test (calculation of *t*, degrees of freedom, *df*) of the significance (probability, *P*) of the differences in incidences of the 4 types of synapse in the pooled data from the ventromedial nuclei of the 3 cyclic and the 3 non-cyclic groups

Type of Synapse		df	t	P
Non-strial	Shaft	34	1.74	ns
	Spine	34	0.08	ns
		<i>n</i> <sub>1</sub> / <i>n</i> <sub>2</sub>	U	P
Strial	Shaft	18/18	158	ns
	Spine	18/18	139	ns

ns, not significant.

treated on day 4, although the incidence of non-strial spine synapses in the preoptic area was 4.9—i.e., in the range of the normal untreated females.

### Mating Behaviour

Tests for female mating behaviour were carried out on the genetic males (for a detailed account see Brown-Grant [9]). When tested as adults with vigorous males the lordosis quotient (LQ) of all groups was between 0 and 4 before hormone treatment. After priming with 2 or 3 daily doses of 50 µg of oestradiol benzoate i.m. the LQs were  $81 \pm 6$  (standard error) for the males castrated within 12 h of birth,  $44 \pm 6$  for the males cas-

trated on day 7, and  $41 \pm 7$  for the males castrated as adults. Following the treatment, 1.25 mg of progesterone were administered and 4–6 h later it was found that the LQs of the males castrated within 12 h of birth had risen to  $100 \pm 0$ ; this response is comparable to that of a normal female. The LQs of the males castrated as adults failed to rise at all ( $40 \pm 4$ ), and the LQs of the males castrated on day 7 showed an intermediate rise ( $66 \pm 9$ ).

### Discussion

The hypothalamic components of the stria terminalis arise principally from the corticomedial group of amygdaloid nuclei (21, 58). For the quantitative analysis of synapses we have selected those parts of the preoptic area and ventromedial hypothalamic nucleus in which these axons establish synaptic contacts (21, 34, 47). Typically the cells of the strial part of the preoptic area have few, beaded dendrites which have very few spines and which are orientated medially and laterally from the cell body so as to intersect in a rectilinear lattice with the strial axons. Lateral to this region and separating it from the ventral part of the bed nucleus of the stria terminalis lies a compact cluster of cells, which we have referred to as the round nucleus; it probably corresponds to subdivision (a) of the medial preoptic area in Gurdjian's account (41). Strial fibres

establish synapses in the strial part of the preoptic area and in the strial bed nucleus, but not in the round nucleus (see also figure 31.14 in ref. 21).

Our observations with the fluorescent technique for catecholamines confirm the reports of a dense fluorescent fibre plexus in the ventral part of the strial bed nucleus (36). These fibres are reported to be noradrenergic (36), to arise from specified cell groups in the medulla and pons (69) and to ascend to the preoptic area through the medial forebrain bundle (3). The present observations show that these fibres extend medially from the strial bed nucleus into the ventral edge of the strial part of the preoptic area, passing ventral to the round nucleus which is itself free of fluorescent fibres. This association of aminergic fibres with an area of presumed involvement in the control of ovulation and possibly mating behaviour is interesting in view of the evidence that biogenic amines are important in reproductive functions, especially since most work has so far been directed towards the tuberoinfundibular system of dopaminergic neurones (38, 81), and much less attention has been paid to the possible involvement of other aminergic systems (e.g., ref. 53).

An important finding to emerge from the quantitative ultrastructural analysis of synapses is that the number of non-degenerating (i.e., non-strial) spine synapses per unit area in the preoptic area is about twice as great in the female (5.3) as in the male (3.3). No such difference between the sexes exists for the non-strial shaft synapses or for either the strial shaft or spine synapses. The suggestion that the sexual difference is specific to the strial part of the preoptic area is supported by the observation that there is no sexual difference in the incidence of any of the 4 types of synapses in the ventromedial nucleus, an area of special value as a control since it too contains strial synapses and may also be related to the control of gonadotrophins. A similar quantitative study of the projection of the fimbria to the septal nuclei (75) also failed to reveal any sexual dimorphism. Functionally it has been suggested that dendritic spines provide an excitatory synaptic mechanism (27, 28) of importance in such functions as the fine temporal discrimination of inputs (23).

It has to be stressed that in its frame of reference the present study is highly selective, and even though the strial part of the preoptic area has been defined within close limits, this does not mean that such an area is necessarily homogeneous in either its cell types or connections. It would be of maximum interest to know what is the source of the non-amygdaloid axon terminals, especially the sexually differentiated ones. Some proportion of these may be derived from intrinsic axons or axon collaterals of the type described by Valverde (93). Furthermore there is no direct anatomi-

cal data indicating the precise projection field of the axons of the cells which lie in the strial part of the preoptic area. It is likely that they are connected, fairly directly (13, 25, 90), with cells in the arcuate-ventromedial region whose axons form terminals in the median eminence—i.e., with the neurones of the parvicellular neurosecretory system (84).

The incidence of spine *synapses* at the ultrastructural level cannot be directly correlated with the incidence of dendritic *spines* in the Golgi material. Apart from the possibility that some of the spines seen in the electron microscope may be too small to be recognised in the Golgi material, there are at least 3 possible ways in which the lower number of spine *synapses* in the male could be achieved: (i) a change in the relative proportions of the spiny dendrites as opposed to the beaded, spine-poor dendrites; (ii) a decrease in the density of spines borne by either or both of these categories of dendrite; and (iii) no actual difference in the number of *spines* but a change in the number of axon terminals making contact with each of a fixed number of spines.

There have been several reports of different types of sexual differentiation in various parts of the central nervous system. These include neuronal nuclear size in the preoptic area and ventromedial nucleus (24, 70), various biochemical differences (e.g., in oxidative enzymes or 5-HT) in the hypothalamus (12, 57, 65), ultrastructural differences in axon terminals in the arcuate nucleus (76), differences in oestrogen retention and responsiveness in the hypothalamus (35), spinal sexual reflexes (46), or even differences in the numbers of preganglionic sympathetic neurones in the spinal cord (11). This raises the question: to what extent can sexual dimorphism in particular sites in the brain be related to particular functions? The evidence supporting the suggestion that the spine synapse difference in the strial part of the preoptic area is correlated with sexually dimorphic reproductive functions is of two types (which will be considered in order): firstly, evidence that the anatomical difference occurs in the same part of the brain as has been implicated in these functions; secondly, evidence that the anatomical difference undergoes neonatal differentiation under the influence of androgen (and independently of genetic sex) in the same way as the functional differences.

#### (1) Correlation of the Morphological Observations with the Evidence for the Localisation of Reproductive Functions

Localised electrical, electrochemical or chemical stimulation of the brain can be used to map out the regions concerned in the control of gonadotrophic hormone release (16, 55) and ovulation (29, 33). In the female rat stimulation of the arcuate nucleus, the medial preoptic area or the amygdala can induce ovulation. The effects of preoptic stimulation are prevented by bilateral cuts

separating this area from the tuberal hypothalamus (87) and the effects of amygdaloid stimulation are prevented by sectioning the stria terminalis (95). This suggests that these structures may be functionally linked in the following way: amygdala → stria terminalis → medial preoptic area → tuberal hypothalamus. By surgically isolating an "island" of tissue from the rest of the brain Halász (42) demonstrated that the tuberal hypothalamus (containing the arcuate nucleus and the ventromedial part of the ventromedial nucleus, i.e., specifically the part sampled in this study) is capable of the maintenance of a basal secretion of gonadotrophic hormones in both males and females, but the females did not ovulate (see also refs. 64, 85). Our anatomical observations show that there is no sign of sexual dimorphism in that part of the tuberal hypothalamus receiving synapses from the stria terminalis.

Halász (42) further demonstrated that ovulation can occur in the female rat if the "island" of tissue was extended forward so as to include the preoptic area. This suggests that the preoptic area is capable of triggering the ovulatory surge of gonadotrophins which is characteristic of the female brain and absent in the male, and this trigger may be correlated with the observation of a higher incidence of spine synapses in the female than in the male.

Direct stimulation by electrodes implanted in the preoptic area can cause ovulation not only in normal female rats but also in females rendered anovulatory by neonatal androgen administration (32, 88) and in male rats bearing ovarian grafts (71). Thus although the preoptic area in these latter two groups of animals is incapable of a spontaneous induction of ovulation, it is capable of inducing ovulation if stimulated appropriately. On this evidence Everett (31) suggested that it is some afferent connection of the preoptic area which is sexually differentiated and this fits well with the present observation of a sexually dimorphic category of synapse. Velasco and Taleisnik (95) and Arai (4) have shown that stimulation of the amygdala leads to a rise in plasma gonadotrophin levels or ovulation in the normal female but not in the neonatally androgen treated female or the male. This demonstrates that the amygdala has access to a sexually differentiated neural mechanism located at the level of the preoptic area. Our observations show that it is not the terminals of amygdaloid origin themselves which are sexually dimorphic. The fact that ovulation can occur after rostral, lateral and dorsal deafferentation of the preoptic area (42, 54, 86) or bilateral destruction of the stria terminalis (10) shows that the amygdaloid input to the preoptic area is also not essential for ovulation.

It is important to compare as closely as possible the precise location of the area we have found to be sexually dimorphic with what information is available from

functional studies as to the localisation of reproductive functions in the preoptic region and also such data as the localisation of the uptake of tritiated oestrogen (83) and the position of cells which show an increased firing rate during the pro-oestrous stage of the cycle (26, 56, 66, 91).

Everett and co-workers (30, 32, 33, 51) have plotted the location of sites from which direct electrical or electrochemical stimulation can induce ovulation in rats in which spontaneous ovulation has been blocked by barbiturates. In their studies the most effective sites appeared to be in the region of the nucleus of the diagonal band, although Terasawa and Sawyer (89) found that the threshold in the preoptic area was lower. Examination of the typical electrode locations illustrated in figure 31.5A, B of their paper (see also ref. 90) shows that the electrode tips were located very close to the sexually differentiated area of the present study.

Hillarp (48) showed that the production of an anovular state with persistent vaginal cornification required destruction of quite a large area of tissue at the level of the optic chiasma, whereas further caudally it could be produced by bilateral destruction of a much smaller part of the ventral hypothalamus. He suggested that the fibres concerned with the induction of ovulation arise from a somewhat diffuse rostral region and are funnelled into compact tracts as they pass backwards and ventrally towards the tuberal hypothalamus; an essential component of the lesion is destruction of the ventral part of the preoptic–anterior hypothalamic junctional area (6). In a preliminary series of experiments we have found that in some cases at least rats with complete bilateral destruction of the strial part of the preoptic area can ovulate, although more subtle changes in endocrine function have not been excluded (unpublished). It is therefore possible that the sexually dimorphic area as defined in the present morphological study is only part of a larger area, all of which is concerned with the control of ovulation.

## **(2) Correlation of the Morphological Evidence with the Evidence for the Neonatal Differentiation of Functions by Androgen Independently of Genetic Sex**

The results of the neonatal endocrine manipulations show that the incidence of non-strial spine synapses in the strial part of the preoptic area is dependent upon the presence of testosterone during the critical period in exactly the same way as is the ability to maintain a cyclic surge of gonadotrophins (43). The density of non-strial spine synapses in the intact female ( $5.3 \pm 0.3$ ) is comparable to that of the males castrated within 12 h of birth ( $5.0 \pm 0.3$ ) and to that of the females treated with testosterone on the 16th day of life ( $5.4 \pm 0.5$ ). Conversely, the male pattern ( $3.3 \pm 0.2$ ) is found in the intact and adult castrated

males, in the females treated with androgen on the 4th day of life ( $3.5 \pm 0.2$ ) and, although to a lesser degree, in the males castrated on the 7th day of life ( $3.9 \pm 0.4$ ).

The questions as to the site and mode of action of androgen (15, 35, 67, 68) are difficult to answer since so little is known about the detailed anatomical development of the preoptic area during the critical period when differentiation is occurring (52). The fibres of the stria terminalis are recognisable from as early as the 17th day of foetal life in the rat (17) but even in Nissl stained sections it can be seen that the preoptic area is quite immature at the time of birth. In various parts of the central nervous system it has been shown that processes such as neurogenesis (2), synaptogenesis (1, 61), the development of dendritic spines (80) and the maturation of the aminergic fibre systems (7, 53, 60) continue during and after the first two weeks of postnatal life and the formation of the adult number and pattern of spines and of synapses can be modified by environmental and endocrine factors (39, 62, 77, 78) acting postnatally.

With regard to the differentiation of the cyclic pattern of gonadotrophin release, our own observations on the vaginal smear patterns show that the females treated with androgen on day 4 were acyclic, with more than 70% cornified smears and polyfollicular ovaries at autopsy. With one exception the females treated with androgen on day 16 had normal vaginal cycles and corpora lutea in the ovaries at autopsy. Evidence available from the literature (43) indicates that when transplanted with ovaries, the males castrated within 12 h of birth are capable of maintaining ovulation, whereas those castrated after the critical period, or as adults, are not. With regard to mating behaviour, normal adult females show a marked enhancement of receptivity (as measured by the lordosis quotient) after ovariectomy, oestrogen priming and progesterone administration. This response is abolished by treatment with testosterone on the 4th day of life (9). Similar tests carried out on the various groups of genetic males used in the present experiment showed that the facilitation of lordosis induced by progesterone in oestrogen primed animals occurred in the males castrated within 12 h of birth and to a lesser degree in those castrated on the 7th day of life; it was absent in the males castrated as adults.

In conclusion, the anatomical observation of a high incidence of non-strial synapses on dendritic spines in the preoptic area is correlated with the ability to initiate a preovulatory surge of gonadotrophins and the ability of progesterone to facilitate lordosis; these are all characteristics of normal females and of males castrated within 12 h of birth. Conversely, in normal males and in neonatally androgenised females, there is a low incidence of spine synapses in the preoptic area,

a non-cyclic pattern of gonadotrophin release, and no enhancement of lordosis by progesterone.

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Male and female animals exhibit sex differences in a variety of neural functions, including food intake, the performance of certain types of learning tasks, aggression, and in reproductive behavioral and physiological patterns, which appear to be under the influence of gonadal hormones (Goy and McEwen, '80). Since a number of neuroanatomical sex differences have been identified that are both located in regions of the brain known to influence these functions and that are influenced by gonadal hormones perinatally and/or during adulthood, it is possible that neuroanatomical sexual differentiation under the influence of gonadal steroids in part underlies functional dimorphism. At the ultrastructural level, there are sex differences in dendritic branching patterns of neurons in the medial preoptic area (mPOA) in the hamster (Greenough et al., '77) and the macaque monkey (Ayoub et al., '83), and in the visual cortex (Juraska, '84) and hippocampus (Juraska et al., '85) of the rat in the synaptic organization of the preoptic area (POA) (Raisman and Field, '71), arcuate nucleus (Matsumoto and Arai, '81), and medial amygdala (Nishizuka and Arai, '81) of the rat; and in the neuronal plasma membrane of the arcuate nucleus of the rat (Garcia-Segura et al., '85). In addition, there are dimorphisms in cell groups involved in vocal communication in canaries and finches (Nottebohm and Arnold, '76); in the ventromedial nucleus (VMN) of the hypothalamus (Matsumoto and Arai, '83) and medial nucleus of the amygdala (MeA) (Mizukami et al., '83) of rats; in the mPOA of rats (Gorski et al., '78, '80), gerbils (Yahr and Commins, '83), guinea pigs (Hines et al., '85), ferrets (Tobet et al., '86), quail (Panzica et al., '87); and in the bed nucleus of the stria terminalis (BNST) of guinea pigs (Hines et al., '85) and rats (del Abril et al., '87; Guillaumon et al., '88). Further sex differences have been reported in neural asymmetries of the cerebral cortex (Diamond et al., '79) and the hippocampus (Diamond et al., '82).

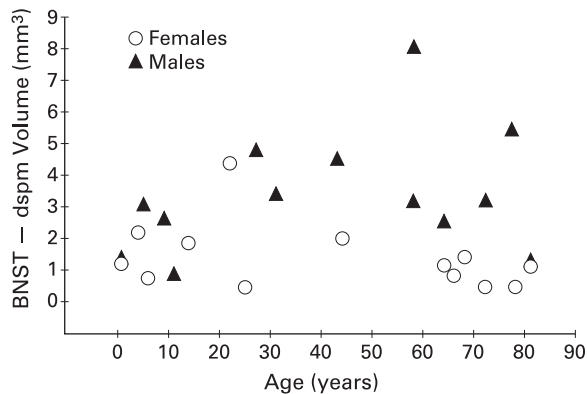
In contrast to the many neuroanatomical sex differences reported in laboratory animals, relatively few have been established in the human brain. However, there is evidence for morphological brain asymmetry

(Wada et al., '75), and at the midsagittal plane of the brain, the massa intermedia is more often present (Rabl, '58) and both the massa intermedia (Allen and Gorski, '87) and the anterior commissure (Allen and Gorski, '86) are larger, and the splenium of the corpus callosum may be more bulbous (de Lacoste-Utamsing and Holloway, '82) in females than in males. There are several sex differences in regions that possibly influence reproductive function: the shape of the supra-chiasmatic nucleus differs between men and women (Swaab et al., '85); Onuf's nucleus in the spinal cord, which innervates perineal muscles, contains more motoneurons in the male than in the female (Forger and Breedlove, '86); and striking dimorphisms exist in cell groups in the preopticanterior hypothalamic area (PO-AHA) (Swaab and Fliers, '85; Allen et al., '89).

Since the BNST exhibits volumetric sex differences in several rodent species, we examined this structure for possible sex differences in the human brain. The only region of the BNST that was darkly stained in our samples was an area in the posteromedial BNST, which appears to be located in a region that, in laboratory animals, exhibits both volumetric (Hines et al., '85; del Abril et al., '87; Guillaumon et al., '88) and neurochemical (DeVries et al., '85; Van Leeuwen et al., '85; Malsbury and McKay, '87) sex differences, appears to concentrate gonadal hormones more heavily than other regions of the BNST (Sar and Stumpf, '75; Stumpf et al., '75; Commins and Yahr, '85; Bonsall et al., '86), and is connected anatomically to other sexually dimorphic nuclei (Weller and Smith, '82; DeVries and Buijs, '83; Lehman and Winans, '83; Simerly and Swanson, '86). Therefore, we evaluated this darkly staining posteromedial region of the BNST (BNST-dspm) for volumetric sex differences in the human brain.

#### Materials and Methods

The brains of 26 human subjects were selected from our brain bank of about 200 limbic forebrain-hypothalamic to represent a range of age-matched male and female pairs (figure 32.1). These brains, collected



**Figure 32.1**

Relations between age and volume of the BNST-dspm. No regression line is illustrated because there was no significant correlation between age and the volume of the BNST-dspm.

from 2 Southern California hospitals on the basis of freedom from neuropathology determined by autopsy and absence of any known neurological disorder according to medical history, were removed from their skulls within 24 hours postmortem, and placed directly into acetate-buffered 10% formalin (ABF) for 2 to 4 weeks prior to coronal sectioning performed during routine autopsy. A block of tissue (approximately 3 cm<sup>3</sup>) passing rostrally to caudally from the diagonal band of Broca to the mammillary bodies, and superiorly to inferiorly from the anterior commissure and anteroinferior columns of the fornix to the base of the brain, was dissected from each selected brain and preserved in ABF for 3 months to 3 years prior to histological preparation. There was no significant difference in duration of fixation between females ( $19 \pm 5$  months) and males ( $18 \pm 3$  months) ( $p = 0.75$ ). Similarly, neither when all subjects were examined as a group ( $r = -0.33$ ;  $p = 0.1$ ) nor when females ( $r = -0.31$ ;  $p = 0.30$ ) and males ( $r = -0.49$ ;  $p = 0.09$ ) were examined separately, were there significant correlations between the volume of the BNST-dspm and the duration of fixation. Subsequently, the selected samples were processed for histology in age-matched pairs as follows: (1) immersion in gelatin—tissue was rinsed overnight in cool water to remove ABF, immersed in 5% gelatin at 37°C for 24 hours and in 10% gelatin for 24 hours, embedded in 10% gelatin, which was allowed to solidify at room temperature, trimmed of excess mold to expose the tissue, and placed in 10% ABF for 4 days to solidify, (2) sectioning—with a sliding microtome, serial, 60- $\mu$ m coronal sections were obtained and mounted from saline onto gelatin-coated glass slides and incubated at 37°C overnight, (3) staining—sections were dehydrated with alcohol, defatted with xylene, rehydrated with distilled water, stained with 1% buffered thionin, rinsed with distilled

water, differentiated with ABF followed by alcohol, and coverslipped out of xylene.

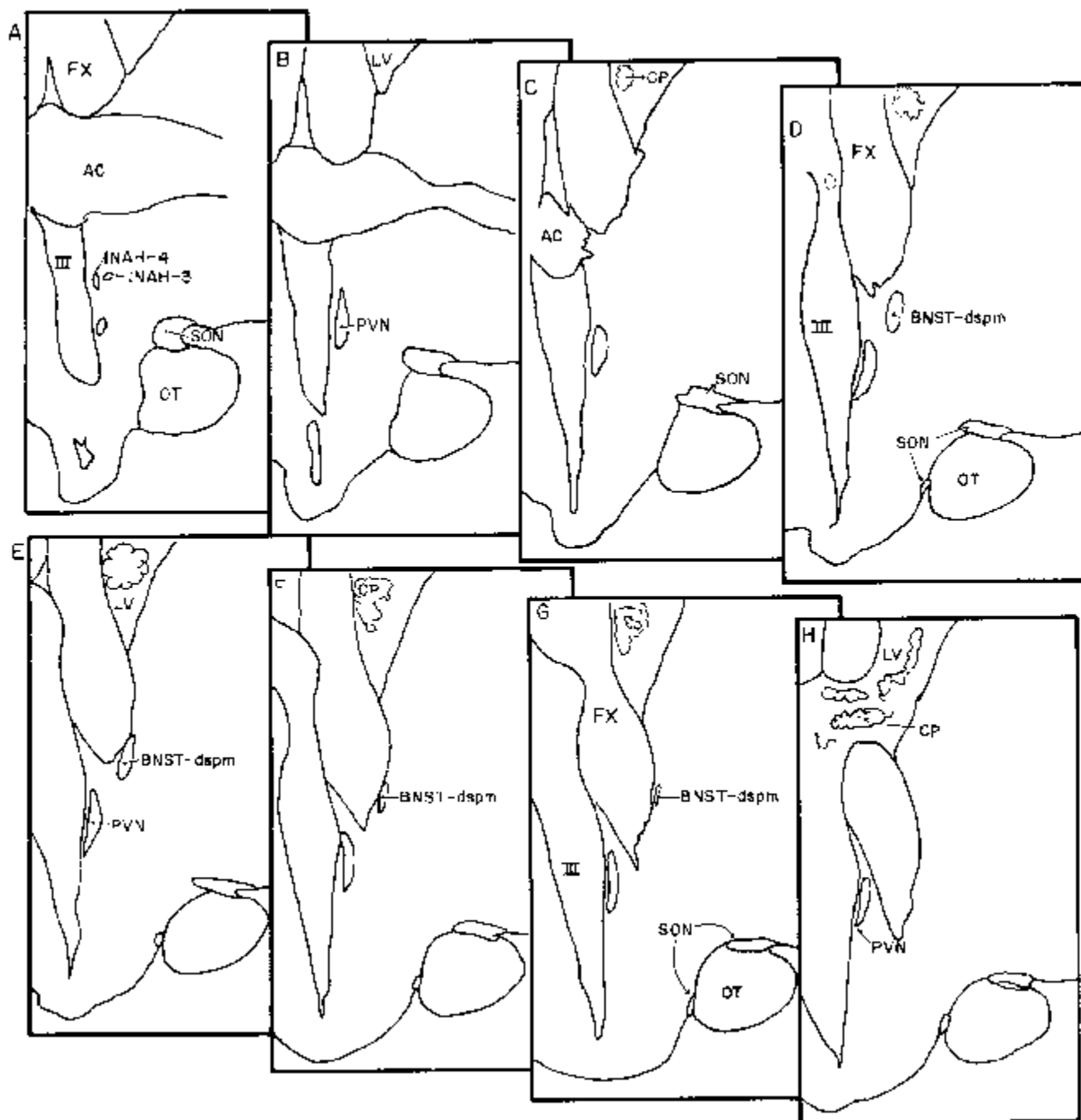
The sections containing the posteromedial BNST were examined. In this region, the most consistent and intensely staining region was located inferolateral to the tip of the fornical column as it enters the hypothalamus (figures 32.2, 32.3). Serial sections of this area were projected onto paper at 44X magnification, and the boundaries of the BNST-dspm were traced independently by 2 investigators who had no knowledge as to the sex of the subjects. By means of other anatomical landmarks, the 2 tracings for each section were superimposed to verify that each investigator had identified in their tracing the same region. In general, there was close agreement between the two investigators; however, if the 2 tracings of a section were not similar, each investigator redrew that section and the 2 drawings, 1 from each investigator, in closest agreement were used. Subsequently, the area of each outline was determined by using a Bioquant Hipad digitizer (Bioquant IBM Program version 2.1; R & M Biometrics), which has a resolution of 0.5 mm and corrects for magnification. The volume of the BNST-dspm was calculated by summing the areas of these values and multiplying by the thickness of the sections.

Differences in volumes were examined using the paired *t* test, and correlations were determined using the Pearson's correlation coefficient.

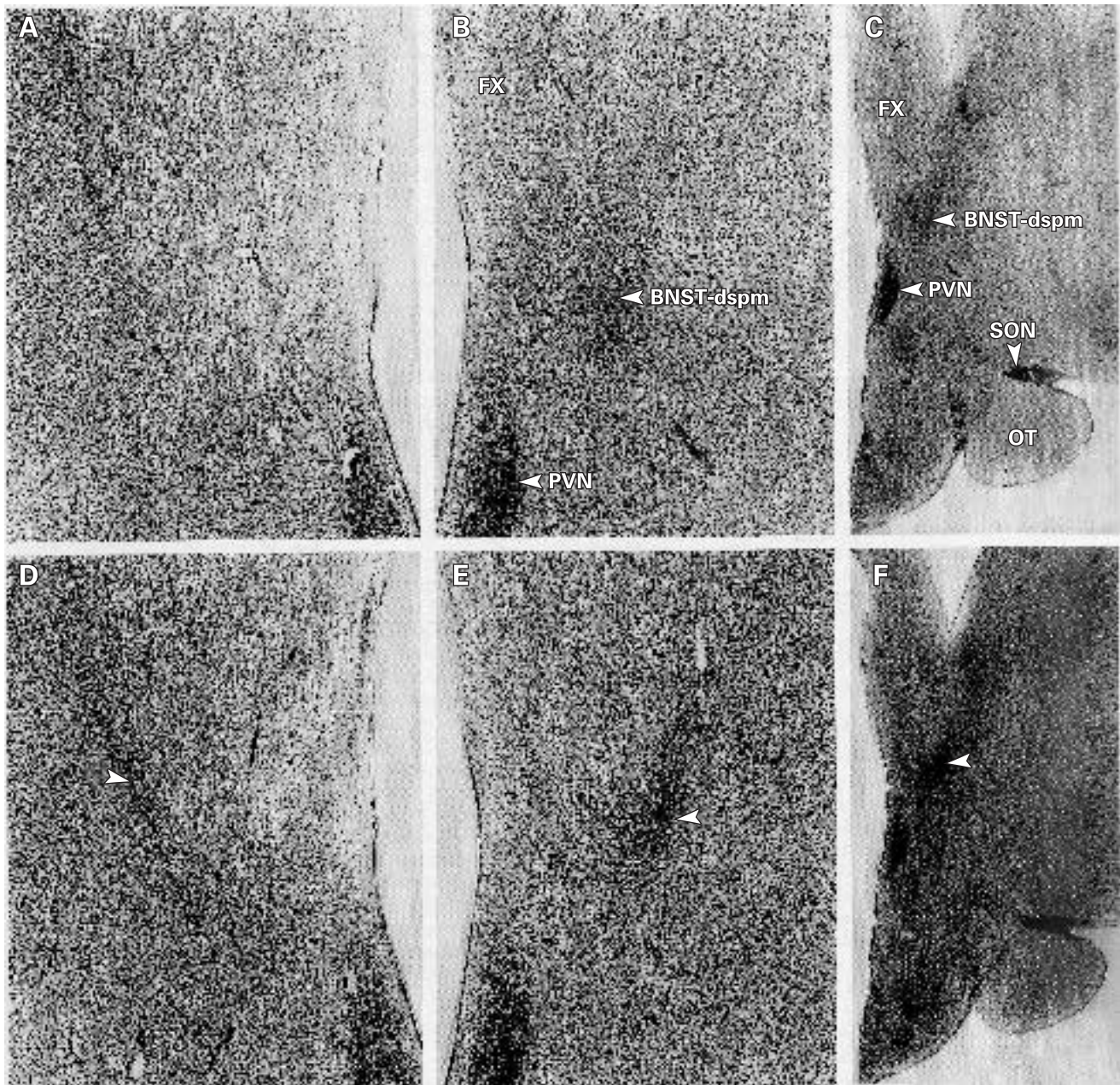
## Results

Our results are based on an analysis of the brains of 13 age-matched pairs of males and females (table 32.1, figure 32.1). The age range for both male and female subjects was 0.8 to 81 years (males: mean  $\pm$  SEM =  $41.3 \pm 8.0$ ; females:  $41.9 \pm 8.5$ ) with an average absolute age difference between pairs of 3 years. There was an average of 10.3 sections per male and 6.0 sections per female BNST-dspm. Although all brains were sectioned coronally during autopsy, the precise plane did vary slightly from subject to subject; therefore, the number of sections did not always correspond to the volume of the nucleus. There was a significant correlation between the tracings of each investigator for both sides of the brain and in each gender (for each comparison:  $r > 0.8$ ;  $p < 0.001$ ). Therefore, subsequent analysis involved the averaged volume obtained by the two drawers.

No hemispheric asymmetries in the volume of the BNST-dspm were observed for either gender (figure 32.4). Among females, the left BNST-dspm ( $1.336 \pm .284$  mm<sup>3</sup>) was slightly greater than the right ( $1.393 \pm .322$  mm<sup>3</sup>) ( $p = 0.73$ ); among males, the left ( $3.346 \pm .598$  mm<sup>3</sup>) was slightly smaller than the right ( $3.395 \pm .557$  mm<sup>3</sup>) ( $p = 0.91$ ).

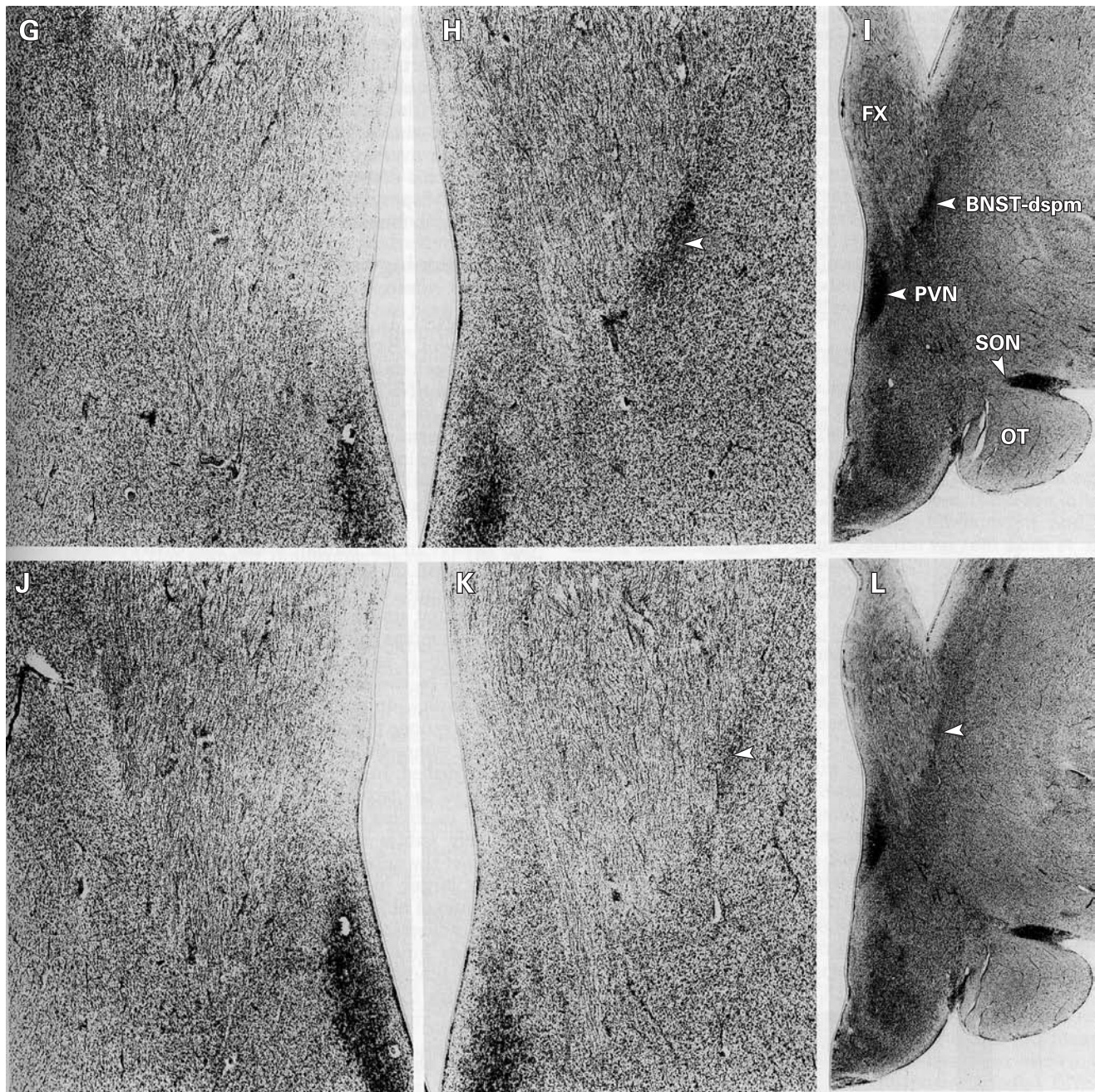
**Figure 32.2**

Schematic illustration of the region analyzed in this study in the coronal plane, organized rostrally to caudally. This atlas was drawn from sections, all projected at the same magnification, from the 64-year-old man (table 32.1). There are seven 60- $\mu$ m-thick sections between each section from A–D and G–H, and one 60- $\mu$ m-thick section between each section from D–G. Levels D, E, F, and G correspond to figure 32.2c, f, i, l, respectively.



**Figure 32.3**

These photomicrographs of thionin-stained coronal sections, located in the region of the anterior hypothalamus, include the darkly staining region of the bed nucleus of the stria terminalis (BNST-dspm), which we found to be sexually dimorphic. Figure 32.2*a, d, g, j*, from the brain of a 68-year-old female, is closely matched with figure 32.2*b, e, h, k*, respectively, from the brain of a 64-year-old man. Figure 32.2*b, e, h, k* is a magnification of figure 32.2*c, f, i, l*, respectively. Figure 32.2*a-c, d-f, g-i, and j-l* is ordered in a rostral to caudal direction, with a 60- $\mu$ m-thick section between each photomicrograph.

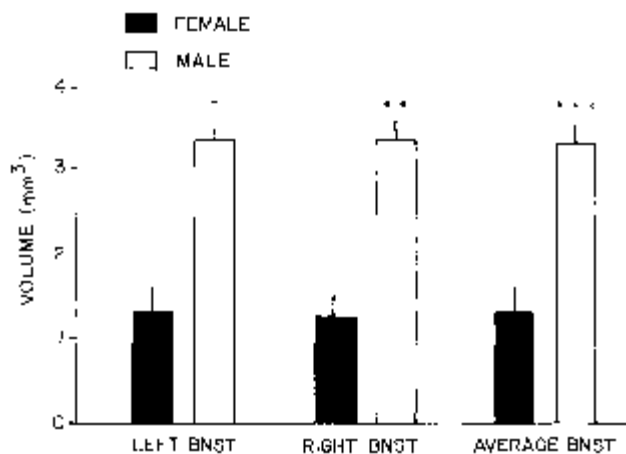


**Figure 32.3**  
(continued)

**Table 32.1**

Individual data on the darkly staining region of the posteromedial BNST (BNST-dspm), cause of death, and race

Pair	Age (yr)	Brain Weight (gm)	Volume (mm <sup>3</sup> )	Race	Cause of Death
<i>Females</i>					
1	0.8	428	1.180	Oriental	Respiratory distress syndrome
2 <sup>a</sup>	4	1,120	2.154	White	Heart disease
3	6	950	0.711	Hispanic	Schwachman-Diamond syndrome
4	14	1,300	1.800	White	Acute lymphoblastic leukemia
5	22	1,200	4.304	White	Adenocarcinoma
6 <sup>a</sup>	25	1,150	0.410	White	Asplastic anemia
7 <sup>a</sup>	44	1,275	1.929	Black	Sickle cell disease
8 <sup>a</sup>	64	1,190	1.120	White	Cryptogenic cirrhosis
9	66	1,150	0.772	White	Cervical cancer
10	68	1,200	1.363	White	Heart disease
11 <sup>a</sup>	72	1,250	0.435	White	Heart disease
12	78	970	0.450	White	Heart disease
13 <sup>a</sup>	81	1,260	1.107	White	Diabetes mellitus
Mean	41.9	1,111	1.364		
SEM	8.5	64	0.292		
<i>Males</i>					
1	0.8	227	1.396	White	Respiratory distress syndrome
2 <sup>a</sup>	5	1,210	3.029	Hispanic	Acute lymphoblastic leukemia
3	9	1,375	2.591	White	Acute lymphoblastic leukemia
4	11	1,340	0.837	Black	Osteogenic sarcoma
5	27	1,350	4.715	Hispanic	Marfan's syndrome
6 <sup>a</sup>	31	1,490	3.334	White	Chronic myelogenous leukemia
7 <sup>a</sup>	43	1,400	4.434	Hispanic	Neuroadenoma
8 <sup>a</sup>	58	1,195	7.977	White	Heart disease
9 <sup>a</sup>	58	1,450	3.121	White	Adenocarcinoma
10	64	1,100	2.490	White	Leukemia
11 <sup>a</sup>	72	1,420	3.148	White	Heart disease
12	77	1,400	5.386	White	Heart disease
13 <sup>a</sup>	81	1,380	1.364	White	Adenocarcinoma
Mean	41.3	1,257	3.371		
SEM	8.0	91	0.532		

<sup>a</sup>Subjects used in a previous study (Allen et al., 89).**Figure 32.4**

Volume of the BNST-dspm on the left and right side of the brain, and the average volume of the left and right side, in males and females. Data are means  $\pm$  SEMs; \*,  $p = 0.012$ ; \*\*,  $p = 0.003$ ; \*\*\*,  $p = 0.005$ .

The volume of the BNST-dspm was significantly greater in males than in females (table 32.1; figures 32.1, 32.3, 32.4). The left BNST-dspm was 2.50 times greater ( $p = 0.012$ ), whereas the right was 2.44 times greater ( $p = 0.003$ ) in males than in females. When the volumes of the left and right BNST were averaged for each sex, the BNST-dspm of the male ( $3.371 \pm .532$  mm<sup>3</sup>) was 2.47 times greater than that of the female ( $1.365 \pm .292$  mm<sup>3</sup>) ( $p = 0.005$ ).

The sexual dimorphism in the BNST-dspm is not due simply to a sex difference in brain size. The brain weight of males ( $1,256 \pm 91$  grams) was only 13.0% greater than that of females ( $1,111 \pm 64$  grams) ( $p = 0.018$ ), in contrast to the volume of the BNST-dspm, which was 147% greater in males than females, and the brain weight corresponded moderately to the volume of the BNST-dspm ( $r = 0.35$ ;  $p = 0.078$ ). Furthermore, there are other nuclei in this region, such as the supraoptic nucleus and interstitial nuclei of the anterior hypothalamus 1 and 4 (INAH-1 and INAH-4),

which were not sexually dimorphic when quantified in some of these same subjects in a previous study (Allen et al., '89). In table 32.1, subjects that had the supraoptic nucleus and INAH 1 through 4 quantified in the previous study are denoted by an “\*”; therefore, it is probable that the sexual dimorphism of the BNST-dspm represents a unique sexual dimorphism in this region of the brain.

## Discussion

### Methodological Considerations

Although we matched our subjects for age, we could not match them for either the postmortem period prior to placing the brains in fixative or for the duration of fixation prior to histological preparation. These two variables could effect tissue quality or influence shrinkage; however, the postmortem period was less than 24 hours, and there was no significant difference in duration of fixation between male and female subjects, or a relationship between the volume of the BNST-dspm and time in fixative for either males, females, or all subjects combined. Therefore, although either of these variables may have introduced error in our measurements, it is unlikely that they contributed significantly to the sex difference.

Most reports indicate that the human male brain is significantly larger than that of the female by approximately 10% (de Lacoste-Utamsing and Holloway, '82; Allen et al., '88); similarly, in this study, the brains of males weighed an average of 13% more than those of females. However, this does not underlie the sexual dimorphism of the BNST-dspm, which was 147% greater in volume in males than in females.

### Homology of the BNST-dspm to Nuclei of Other Species

The BNST is a heterogeneous nucleus in terms of development (Bayer, '87), cellular structure (McDonald, '83), neurochemistry, connectivity, and function. The region of the BNST that we quantified appears to be located in an area first described in rats as the “special nucleus of the stria terminalis” (Johnston, '23), and, subsequently, the “encapsulated portion” of the BNST in rabbits (Young, '36), and rats (Simerly and Swanson, '87). The special nucleus of the stria terminalis, or the encapsulated portion of the BNST (BNSTenc), is located within the posteromedial BNST. In the human brain, the BNST-dspm, located inferolateral to the tip of the fornix as it enters the hypothalamus, was the only region within the posteromedial area that stained darkly and appeared to be located in a similar region as the BNSTenc of rodents.

Although it is unknown whether the BNST-dspm is analogous to the sexually dimorphic posteromedial BNST of laboratory animals, the posteromedial BNST

is the region in guinea pigs (Hines et al., '85) and rats (del Abril et al., '87) where dramatic volumetric sex differences have been described. In both laboratory animals and humans the volume of the sexually dimorphic posteromedial BNST is greater in males than in females. In contrast, in rats, relatively subtle sex differences have been observed in regions of the anterior and posterolateral BNST, which are slightly larger in the female brain (del Abril et al., '87; Guillamón et al., '88).

Autoradiographic studies indicate that although gonadal hormones bind to neurons throughout the BNST, the greatest binding occurs in the posteromedial BNST in guinea pigs (Sar and Stumpf, '75), gerbils (Commins and Yahr, '85), rats (Stumpf et al., '75), and monkeys (Bonsall et al., '86).

In rats, the sexually dimorphic region of the BNST is specifically connected to several other sexually dimorphic cell groups that bind gonadal steroids, including afferents from the volumetrically dimorphic MeA (Weller and Smith, '82; Lehman and Winans, '83) and efferents to the volumetrically dimorphic medial preoptic nucleus (MPN) (Simerly and Swanson, '86) and the chemically dimorphic lateral septal nucleus (De Vries and Buijs, '83). It remains to be determined whether other afferents to the BNST from the amygdala (Krettek and Price, '78; Scalia and Winans, '75; Swanson and Cowan, '79), supramammillary region (Shepard et al., '88), peripeduncular nucleus (López and Carrer, '82), ventral subiculum, VMN, and brainstem aminergic cell groups (Swanson and Cowan '79); and other efferents from the BNST to the amygdala (Morrell et al., '84; Swanson and Cowan, '79), ventral tegmental area of Tsai (Morrell et al., '84), laterodorsal tegmental nucleus (Sato and Fibiger, '86), paraventricular nucleus (Swanson and Cowan, '79; Saphier and Feldman, '86), VMN (López and Carrer, '82), substantia innominata, nucleus accumbens, POA, central tegmental fields of the midbrain, ventral tegmental area, dorsal and median nuclei of the raphe, locus coeruleus, anterior nuclei of the thalamus, parataenial nucleus, and medial habenular nucleus (Swanson and Cowan, '79) involve cells of the sexually dimorphic region of the BNST.

In addition to interconnections between the BNST and other sexually dimorphic nuclei, there appear to be interconnections between many nuclei that bind gonadal hormones, are involved in sexually dimorphic reproductive function, and some exhibit volumetric and neurochemical dimorphism, suggesting the existence of sexually dimorphic neural circuits. In rats, recent attention has been placed on the role of the BNSTenc in the sexually dimorphic neural circuit integrating olfactory information, in which the MeA sends projections to the BNSTenc (Scalia and Winans, '75)

and, in turn, the BNSTenc projects to the MPN (Simenly and Swanson, '86). In the human brain, sex differences are present in the PO-AHA (Swaab and Fliers, '85; Allen et al., '88) and in the BNST, although no sex differences were observed in the MeA (Murphy, '86).

Although it is unclear whether the BNST-dspm is analogous in terms of sexual dimorphism, steroid binding, and connectivity to the sexually dimorphic region of the posteromedial BNST in laboratory animals, specific neurochemical characterization in both laboratory animals and in humans may elucidate this possibility. The posteromedial BNST has been characterized immunohistochemically in rats for the sexually dimorphic distribution of vasopressin- (DeVries et al., '85; Van Leeuwen et al., '85) and CCK-immunoreactive cell bodies and substance P-like immunoreactivity (Malsbury and McKay, '87); and the medial BNST of the hamster exhibits a sex difference in the pattern and density of opiate receptors (Ostrowski et al., '87). It is probable that upon further examination, additional sex differences will be observed in the other neurochemical characteristics of this region. For example, the posteromedial BNST in rats contains enkephalin- (Finley et al., '81), vasoactive intestinal polypeptide- (Roberts et al., '80), and galaninlike (Melander et al., '86) immunoreactive cell bodies;  $\beta$ -endorphin- (Finley et al., '81), enkephalin- (Finley et al., '81), vasoactive intestinal polypeptide- (Roberts et al., '80) and galaninlike (Melander et al., '86) immunoreactive fibers; binding sites for oxytocin (Mercier et al., '87), galanin (Skofitsch et al., '86), and corticotropin releasing factor (Wynn et al., '84); and proenkephalin mRNA-containing neurons (Harlan et al., '87). Although each of these neurotransmitters has not been evaluated for their presence in the posteromedial BNST of humans, let alone the BNST-dspm, several neurotransmitters have been identified in the posteromedial BNST of the human brain: pro-somatostatin-derived-peptide-positive fiber tracts (Bouras et al., '87), a dense dopaminergic input (Gaspar et al., '85), and neurotensin- (Mai et al., '87) and vasopressin-immunoreactive cell bodies (Fliers et al., '86).

### Function

The BNST is involved in several functions that appear to be sexually differentiated. Electrical stimulation of the BNST modulates aggressive behavior in cats (Shaikh et al., '86), and corticosterone secretion in rats (Dunn, '87). In addition, the BNST has been shown to play an important role in behavioral and neuroendocrine aspects of reproduction. Specifically, lesions of the BNST decreased male copulatory behavior in rats (Emery and Sachs, '76; Valcourt and Sachs, '79) and hamsters (Powers et al., '87), and impaired chemoinvestigative behavior in hamsters (Powers et al., '87).

In female rats, chemical (Velasco and Taleisnik, '69) and electrical (Kawakami et al., '70) stimulation of the BNST has been shown to induce ovulation, and estrogen implantation (Terasawa and Kawakami, '74) and electrical stimulation may induce or inhibit gonadotropin secretion, depending on location (Beltramino and Taleisnik, '80) and time during the estrous cycle (Kawakami and Kimura, '74) that the stimulus is applied. However, it remains unclear what specific role the volumetrically sexually dimorphic region of the BNST of rodents plays in these sexually differentiated functions, and even less is known in humans.

### What Determines the Sexual Dimorphism?

We do not know whether genetic, environmental and/or gonadal hormones influence the sexual dimorphism of the BNST-dspm. Whereas some sex differences in rodents appear to be influenced by environmental factors (Juraska, '84; Juraska et al., '85; Berrebi et al., '88; Juraska and Kopcik, '88), most sexually dimorphic structures are influenced by gonadal hormones during development and/or during adulthood. For example, in the rat (del Abril et al., '87) and guinea pig (Hines et al., '85), perinatal gonadal hormone levels appear to determine the volume of the BNST. In other cases, such as the sexually dimorphic area of the gerbil (Commins and Yahr, '84), nuclei involved in song behavior in canaries (Nottebohm, '80, '81), and the sexually dimorphic nucleus of the preoptic area in rats (Gorski et al., '78, '80; Bloch and Gorski, '88), volumetric sex differences are determined both by perinatal as well as adult steroid hormone levels. The sexual differentiation of a nucleus in the human brain has been reported to begin after 2 to 4 years of age (Swaab and Hofman, '88). The mechanism by which gonadal steroids influence neural structure is currently under investigation. However, sex hormones have been shown during development to prevent normally occurring neuronal death (Nordeen et al., '85) and to increase neurite outgrowth (Toran-Allerand, '80; Toran-Allerand et al., '80). In the adult brain, gonadal steroids may play a powerful role in neural plasticity by inducing dendritic growth and the formation of functional synapses (DeVoogd and Nottebohm '81; Matsumoto and Arai, '81). Accordingly, each volumetrically sexually dimorphic cell group in each species examined for gonadal hormone-binding neurons by autoradiography has been shown to exhibit labeling. The fact that the posteromedial BNST contains both the heaviest steroid labeling as well as the sexually dimorphic component of the BNST in several species examined thus far suggests further the role of gonadal hormones in the sexual differentiation of the volume of this dimorphic nucleus. Although histological analysis has not been performed on gonadal hormone binding in the human brain, it is probable that relatively intense steroid hormone labeling will

characterize the BNST-dspm and other sexually dimorphic nuclei of the human as well.

Although sex differences in the volume of the BNST appear to occur during development in rats (del Abril et al., '87; Guillaumon et al., '88) and not as a result of adult hormone levels in guinea pigs (Hines et al., '85), neurochemical sex differences in the BNST result from both the organizing effects of gonadal hormones during development (de Vries et al., '83; De Vries and Buijs, '83) as well as their activational effects during adulthood (de Vries et al., '85; Simerly and Swanson, '87; Micevych et al., '88a,b). Based on our sample size and age range of subjects (table 32.1, figure 32.1), it is unclear whether the BNST-dspm is sexually differentiated during development and/or whether it is influenced by gonadal hormones or environmental factors later in life. Whereas the role of gonadal hormones on sex differences in the human brain remains unclear, several facets of human behavior may be influenced by hormone levels during both the perinatal period (Ehrhardt and Meyer-Bahlburg, '81; Hines, '82) and adulthood (Reinisch and Haskett, '81).

### Conclusions

There are several reasons why the identification of structural sex differences in the human being may be of importance. For example, during development, factors such as prenatal stress of the mother, which both feminize and demasculinize sexual behavior in rats (Ward, '84) and decrease the volume of the sexually dimorphic nucleus in the preoptic area of male rats (Anderson et al., '86), may, similarly, contribute in human males to homosexuality (Dörner, '76). We can speculate that such prenatal stress may decrease the volumes of sexually dimorphic nuclei in the human brain, which, interestingly, are present in regions of the brain believed to influence gonadotropin secretion, a function that may be altered in some homosexual men (Dörner, '76; Gladue et al., '84). In addition, gonadal hormones may be important neurotrophic factors both during development and in adulthood and, in fact, have been shown to improve symptoms of Alzheimer's disease in some postmenopausal women (Fillit et al., '86). Unlike laboratory animals, the human being cannot be manipulated experimentally; therefore, it will be difficult to elucidate the role of gonadal hormones in sexually differentiating the human brain. However, if we are able to identify regions of the human brain that are sexually dimorphic, we may subsequently examine those structures in subjects who have been exposed to pathological perinatal steroid hormone levels that would occur in people who, for example, have an endocrine anomaly such as congenital adrenal hyperplasia; are of different age groups with corresponding different gonadal hormone levels; have been treated therapeutically as adults with gonadal

hormones; have undergone ovariectomy or orchidec-tomy; or even in individuals such as homosexuals and transsexuals whose sexual preference and orientation, respectively, may have a perinatal hormone basis. Finally, in an attempt to help bridge the missing link in the role of gonadal hormones on influencing neural structure and function between laboratory animals and human beings, it may be possible in the future to correlate sexually dimorphic neural structures with the individuals' history of gonadal hormone exposure and even their sexual preference or orientation.

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## Introduction

The concept of the sexual differentiation of brain function is now well established, particularly with regard to the regulation of gonadotropin secretion (9, 11), male and female sexual behavior (10, 26), the regulation of food intake and body weight (19, 29) and aggressive behavior (24). In brief, this concept assumes that those functions recognized as “masculine” in the adult are at least partially a result of the action of testicular hormones on the developing brain, which is undifferentiated and/or inherently female. Although this concept of a permanent or organizational (23) action of the hormonal environment on neuronal differentiation and development is of considerable importance to both neuroendocrinology and neuroscience in general, the mechanism of this effect is poorly understood.

That protein synthesis may be involved in this permanent action of androgen is suggested by the demonstration that antibiotic administration can block or attenuate its action (12, 17, 27). It has also been proposed that early hormone exposure permanently alters the steroid hormone-receptor systems which are thought to mediate steroid influences on the nervous system (9, 18). More recent evidence suggests the possibility that there exist sexual differences in neural connectivity (7), perhaps with a morphological basis (14, 25). The latter studies suggest that there may be a subtle, but significant difference in the localization of synaptic terminals (25) or in the patterning of dendritic branches (14) within the region of the preoptic area (POA). The POA, in turn, is considered to be a focal point for this organizational action of androgen (9, 11).

In contrast to these subtle morphological differences, Nottebohm and Arnold (20) recently reported the existence of a gross sex difference in the volume of the hyperstriatum ventrale pars caudale and the robust nucleus of the archistriatum, which are involved in the androgen-sensitive control of singing in the canary and the finch. In the present study we investigated the preoptic region of the brain of the rat, an extensively studied model of sexual differentiation, and now report the existence of a striking morphological sexual dimor-

phism which appears to be essentially independent of gonadal hormones in the adult, but which is critically dependent upon the perinatal hormone environment. Portions of this work have been presented in abstract form (13).

## Methods

### Experiment I. Influence of the Hormone Environment in the Adult

Because a preliminary study (11) revealed that the volume of the medial preoptic nucleus was significantly larger in the orchidectomized male than in the ovariectomized female, an extensive analysis of the possible effects of the hormone environment on this sex difference was carried out in the adult rat. The brains of 76 animals were analyzed for this experiment. All rats were gonadectomized as young adults and males and females were divided among the following 6 groups: Group 1, no further treatment and sacrificed 5–6 weeks after gonadectomy. Group 2, sacrificed two weeks after gonadectomy; 0.05 ml sesame oil was injected on the last three days of this period. Group 3, sacrificed two weeks after gonadectomy, 4–6 h after the administration of 500  $\mu$ g progesterone, which followed three daily injections of 2  $\mu$ g estradiol benzoate. This hormone regime markedly facilitates sexual receptivity in the female (10). Group 4, injected with 500  $\mu$ g testosterone propionate daily for the two weeks between gonadectomy and sacrifice. This hormone regime would be expected to maintain sexual behavior in the orchidectomized male rat (8, 33). Group 5, fed 0.15% propylthiouracil (PTU) mixed in the food for one month between gonadectomy and sacrifice in order to disrupt thyroid function. Group 6, two weeks after gonadectomy these animals were deprived of water for 24 h and then sacrificed.

At the time of sacrifice, the animals were weighed, and immediately after death perfused under gravity with saline for one minute and then with 10% formalin in saline for 4 additional minutes. Care was taken to keep the perfusion pressure comparable from animal to animal by maintaining the level of the perfusion

fluid in the reservoir containers. After perfusion, the brains were carefully removed from the cranium, the olfactory bulbs removed, and the spinal cord transected just caudal to the pyramids. The brain was weighed and stored in 10% formalin-saline. In several groups thyroid weights (after perfusion) were also recorded. In an additional 11 rats, the adrenal glands were removed at the time of gonadectomy, but high plasma corticosterone levels ( $19.5 \pm 0.8 \mu\text{g}\%$ ) at the time of sacrifice two weeks later indicated that adrenal rest tissue had negated the surgery and precluded further analysis of the brains of these animals.

The brains were frozen sectioned in the De Groot plane (4) at  $60 \mu\text{m}$  and every section was saved and stained by submersion for 5 min in a 0.1% aqueous solution of thionine at room temperature (pH 5.2, sodium acetate buffer). The brains were analyzed quantitatively in the following manner: The individual brain sections were projected onto paper at a 37-fold magnification. The portion of the medial preoptic nucleus (MPON), which was as intensely stained as the supra-chiasmatic nucleus (SCN), was outlined on paper directly from the projected image. For each animal, three such tracings were prepared independently by three individuals (the same throughout the study) without knowledge of the sex or treatment of the donor. A fourth investigator, again without knowledge of the sex or treatment group, superimposed the three drawings according to other landmarks (e.g., the boundaries of the third ventricle) on a transilluminated glass plate, and the average outline of the darkly staining component of the MPON was traced on high quality vellum. These tracings were then cut out and weighed. From each sheet of vellum at least 5 squares of  $400 \text{ mm}^2$  were also cut out and weighed. This permitted accurate conversion of the weight of the nuclear tracings to volume in cu. mm. The brains of 9 animals were sectioned in the parasagittal plane for illustration purposes only and were not analyzed quantitatively.

To test for the specificity of the sexual dimorphism in the MPON, the volume of the SCN was similarly quantified in most animals. (In a few cases, histological artifacts precluded the collection of these data.) In addition, the following measurements were obtained from one reference section: the greatest width of the septum, the height of the POA (the distance between the ventral border of the anterior commissure and the dorsal border of the optic chiasm approximately 0.5 mm from midline), and POA width (the distance from midline to the lateral border of the optic chiasm). These measurements were taken  $180 \mu\text{m}$  anterior to the posterior border of the decussation of the anterior commissure. In general, this reference section included the sexually dimorphic component of the MPON.

## Experiment II. Influence of the Hormone Environment Neonatally

For this study, the brains of 23 rats were analyzed after the following treatments as neonates: Group 1, 0.05 ml sesame oil injected to females on day 4 of life (day of birth considered day 1). Group 2, the injection of  $90 \mu\text{g}$  testosterone propionate in oil to females on day 4. Group 3, the injection of 1 mg testosterone propionate in oil to females on day 4. Group 4, castration of one-day-old males under hypothermia. Group 5, sham castration of day 1 males under hypothermia. At the time of weaning (day 21), the animals of Group 5 were gonadectomized, while the neonatally castrated males (Group 4) were sham-operated. At 45–50 days of age, the animals of Groups 1–3 were ovariectomized, and all rats were sacrificed 2 weeks later (60–65 days of age) and perfused as described in Experiment I. All histological and analytical procedures were identical to those of Experiment I.

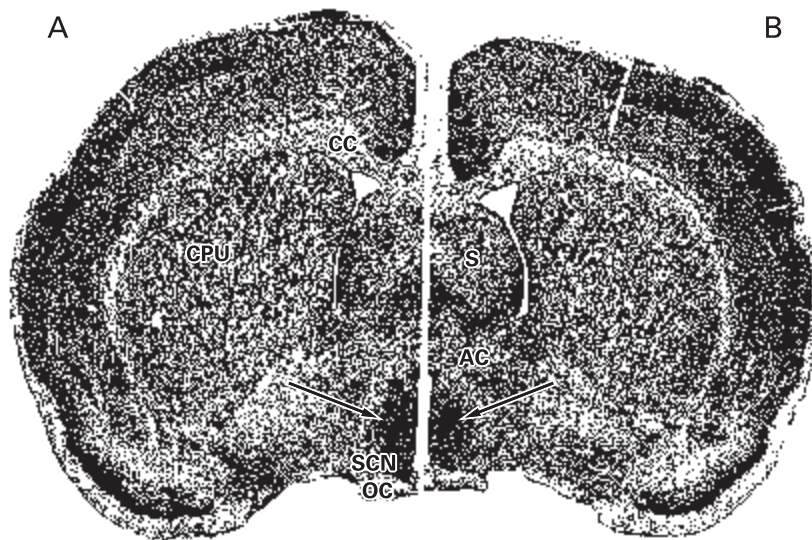
Statistical analyses of all data were performed with computer assistance and involved 1- or 2-way analysis of variance (32) and the Duncan's multiple range test (1). The precise test used to analyze specific comparisons will be identified in the Results section.

## Results

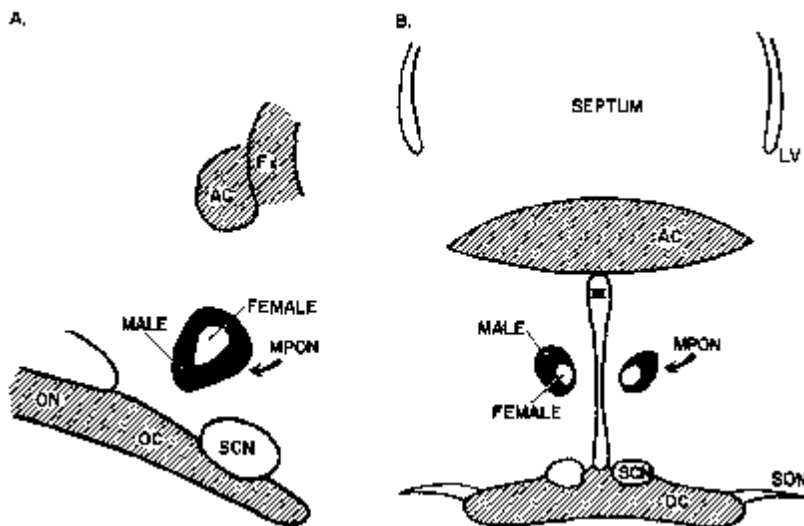
### Experiment I

In confirmation of our preliminary report (11), the volume of the intensely staining component of the MPON was markedly sexually dimorphic; the volume of this nuclear region in the male (0.96 cu. mm) was 8 times greater than that in the female (0.12 cu. mm). In fact, this sexual dimorphism is evident upon observation of brain sections with the naked eye; see figure 33.1, which presents representative sections from one male and one female animal with MPON nuclear volumes close to the mean for the individual groups. Figure 33.2 presents the localization of the MPON in both the male and female. To accomplish this illustration, the histological sections which included the most developed portion of the MPON were superimposed and the average position of the nucleus in each group drawn. In the case of the parasagittal sections, only 4 female brains were available, but in all other groups the general localization of the MPON is based on 5 individual animals. According to these figures, the MPON is approximately 0.6 mm in anterior–posterior diameter, and 0.7 mm in dorsal–ventral diameter, while that of the female is 0.3 and 0.4 mm, respectively.

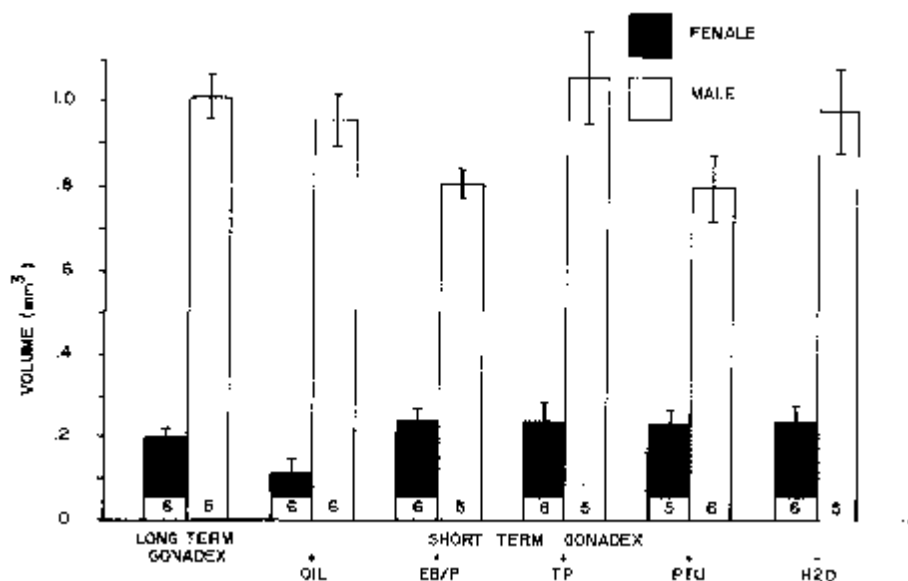
The fact that this sexual dimorphism in the MPON is independent of the hormonal environment in the adult is illustrated in figure 33.3. Two-way analysis of variance (ANOVA) indicated a highly significant

**Figure 33.1**

Coronal sections through the brain of the adult female (*A*) and male (*B*) rat sacrificed two weeks after gonadectomy; thionine. The arrows indicate that portion of the medial preoptic nucleus which exhibits a marked sexual dimorphism. Both at the same magnification. The absence of the supraoptic nucleus (SON) in *B* is an artifact of the plane of tissue sectioning. Abbreviations: AC, anterior commissure; CC, corpus callosum; CPU, caudate putamen; OC, optic chiasm; S, septum.

**Figure 33.2**

Localization of the sexually dimorphic component of the medial preoptic nucleus (MPON) in the sagittal (*A*) and coronal (*B*) planes. The nucleus of the female rat is drawn completely within the volume of the nucleus of the male. Abbreviations: Fx, fornix; LV, lateral ventricle; ON, optic nerve; SON, supraoptic nucleus; III, third ventricle; other abbreviations as in figure 33.1. Drawn from slides magnified 37 $\times$ .



**Figure 33.3**

The persistence of the sex difference in the mean volume  $\pm$  standard error (lines) of the medial preoptic nucleus under different hormonal environments in the adult gonadectomized rat. EB/P, estradiol benzoate/progesterone; TP, testosterone propionate; PTU, propylthiouracil;  $-H_2O$ , water deprivation. See Methods for dosage and duration of treatment. Numbers at the base of each bar represent the number of brains analyzed.

( $P < 0.0001$ ) sex difference but no significant treatment effect; there was, however, a significant ( $P < 0.04$ ) interaction. Further analysis by the Duncan's multiple range test revealed that the mean nuclear volume in any group of males was significantly ( $P < 0.01$ ) greater than any group of females. This test also revealed that the significant interaction lay within the male animals. Although the short-term gonadectomized males (Group 2, which is considered the reference group) was not significantly different from any other group, the nuclear volume in the males of Groups 1, 4 and 6 were significantly ( $P < 0.05$ ) larger than those of the males of Groups 3 and 5. It should also be indicated that the PTU treatment was highly effective in altering thyroid function, as judged by the fact that the thyroid weight in PTU-fed animals ( $114.3 \pm 5.0$  mg) was significantly ( $P < 0.001$ ) greater than that of control animals ( $21.3 \pm 1.3$  mg).

Table 33.1 indicates that this sexual dimorphism in the MPON volume is not a reflection of a sex difference in general brain size or weight. There was no significant sex difference among the groups in preoptic height or septal width. However, two-way ANOVA indicated a sex difference ( $P < 0.04$ ) in preoptic width, which Duncan's multiple range test showed to be due principally to the fact that this parameter in the males of Group 1 was significantly ( $P < 0.01$ ) larger than in any group of females. Although there was no significant difference in brain weight, on the possibility that mean values obscured a possible relationship between brain weight and nuclear volume on an individual basis, the volume of the MPON was expressed rela-

tive to brain weight; but the sex difference remained ( $P < 0.001$ , ANOVA), and in each group of male rats the MPON nucleus was significantly ( $P < 0.01$ ) larger in relative volume than that of any group of females. As in the case of absolute volume of the MPON, statistical analysis revealed no significant treatment effect, but a significant ( $P < 0.02$ ) interaction. Further analysis confirmed that this interaction lay exclusively within the male animals in that the relative nuclear volume of the males of Groups 3 (EB/P) and 5 (PTU) was significantly smaller than that of the males of Groups 1, 4 and 6. Figure 33.4 illustrates the data from a comparable analysis of the volume of the SCN. Two-way ANOVA indicated that there was a significant ( $P < 0.01$ ) sex difference, but no treatment effect and no interaction. Further analysis by the Duncan's multiple range test revealed that the males of Groups 1 and 2 were significantly ( $P < 0.05$ ) greater than those of the females of Groups, 2, 5 and 6. Analysis of the SCN volume relative to brain weight revealed a significant ( $P < 0.02$ ) sex difference which was due to the fact that, even in these relative terms, the males of Group 2 were significantly ( $P < 0.05$ ) different from the females of Groups 2, 5 and 6.

#### Experiment II

The fact that the volume of the MPON is dependent upon the hormone environment during the perinatal period is illustrated in figure 33.5. One-way ANOVA revealed a highly significant ( $P < 0.0001$ ) difference among these groups. Further analysis by the Duncan's multiple range test revealed that the animals of Group

**Table 33.1**

The influence of hormone treatment of the adult gonadectomized male and female rat on brain size and on volume of the medial preoptic nucleus (MPON) and suprachiasmatic nucleus (SCN) relative to brain size

Group	Treatment	Number of Rats	Preoptic Height (mm)	Preoptic Width (mm)	Septal Width (mm)	Brain Wt. (g)	MPON Vol. Brain Wt. (cu. mm/g)	SCN Vol. Brain Wt. (cu. mm/g)
<i>Male</i>								
1	Long-term gonadectomy	5	1.95 ± 0.04	1.57 ± 0.05**	3.13 ± 0.03	1.83 ± 0.05	0.55 ± 0.02	1.19 ± 0.03
2	Oil	6	1.86 ± 0.02	1.25 ± 0.06	3.07 ± 0.03	1.77 ± 0.07	0.54 ± 0.04	1.25 ± 0.06
3	EB/P	5	1.79 ± 0.03	1.30 ± 0.04	3.08 ± 0.04	1.81 ± 0.02	0.45 ± 0.02†	1.08 ± 0.04
4	TP	5	1.94 ± 0.03*	1.34 ± 0.11*	3.10 ± 0.03	1.71 ± 0.05	0.59 ± 0.07	1.14 ± 0.10
5	PTU	6	1.82 ± 0.02	1.19 ± 0.02	3.15 ± 0.05	1.84 ± 0.03	0.43 ± 0.04††	1.09 ± 0.03
6	–H <sub>2</sub> O	5	1.84 ± 0.03	1.28 ± 0.03	3.17 ± 0.04	1.76 ± 0.05	0.56 ± 0.06	1.18 ± 0.07*
<i>Female</i>								
1	Long-term gonadectomy	6	1.85 ± 0.04*	1.25 ± 0.04*	3.16 ± 0.05	1.85 ± 0.01	0.11 ± 0.01	1.07 ± 0.10*
2	Oil	6	1.88 ± 0.03	1.29 ± 0.06*	3.03 ± 0.06	1.77 ± 0.06	0.07 ± 0.02	1.02 ± 0.05*.†††
3	EB/P	6	1.85 ± 0.03	1.25 ± 0.10	2.98 ± 0.07	1.76 ± 0.04	0.16 ± 0.02	1.08 ± 0.06
4	TP	6	1.89 ± 0.01	1.11 ± 0.02***	3.08 ± 0.04	1.72 ± 0.02	0.14 ± 0.02	1.21 ± 0.01
5	PTU	5	1.83 ± 0*	1.19 ± 0.05*	3.10 ± 0.02	1.78 ± 0.03	0.13 ± 0.02*	1.01 ± 0.05*.†††
6	–H <sub>2</sub> O	6	1.90 ± 0.03	1.36 ± 0.06	3.13 ± 0.03	1.76 ± 0.03	0.14 ± 0.02	1.00 ± 0.08†††

Treatment: EB/P, 2.0 µg estradiol benzoate × 3 plus 0.5 mg progesterone; TP, 500 µg testosterone propionate × 14 days; PTU, 0.15% propylthiouracil in ground chow for one month; –H<sub>2</sub>O, water deprivation for 24 h.

\*One less rat in this group.

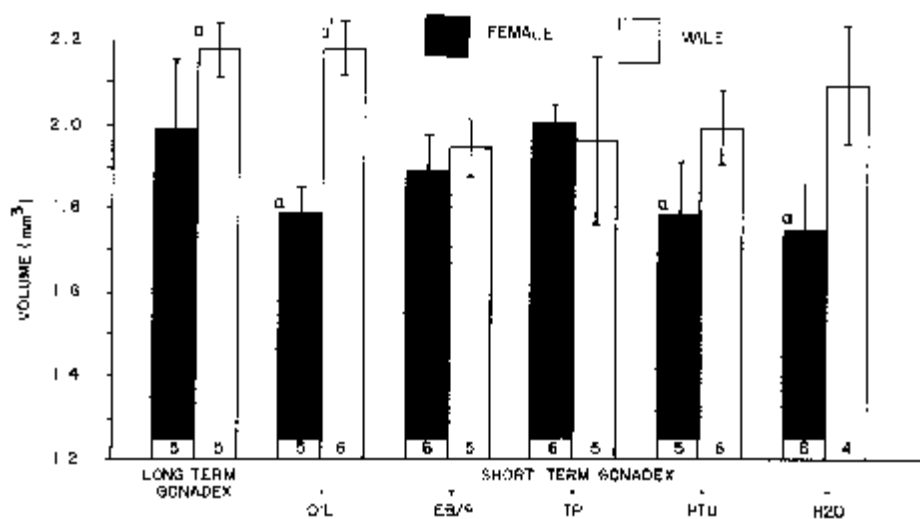
\*\*Significantly ( $P < 0.01$ ) larger than that of any other group.

\*\*\*Significantly ( $P < 0.05$ ) smaller than that of Group 4 males.

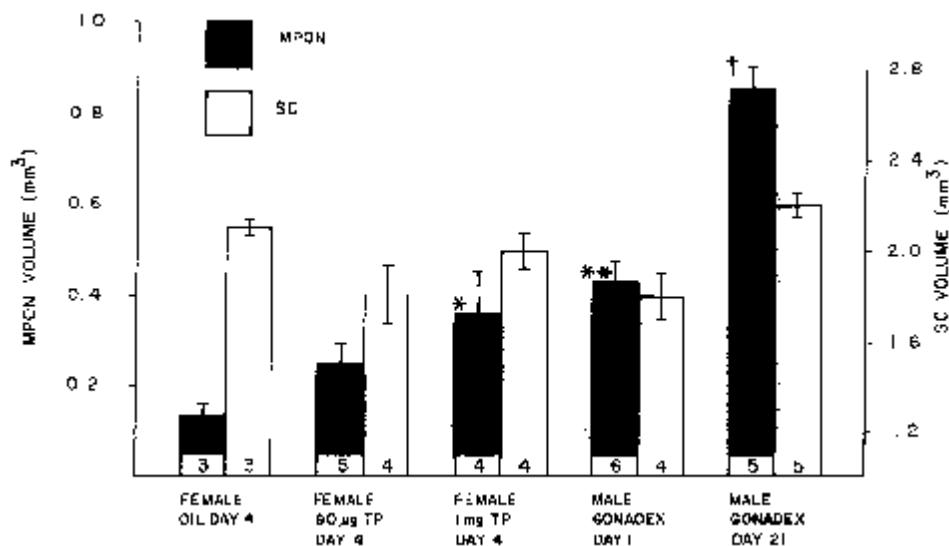
†Significantly ( $P < 0.05$ ) smaller than that of male Groups 1, 4 and 6.

††Significantly ( $P < 0.05$ ) smaller than that of all male groups except Group 3.

†††Significantly ( $P < 0.05$ ) smaller than that of the males of Group 2.

**Figure 33.4**

The influence of hormone treatment of the adult gonadectomized rat on the volume of the suprachiasmatic nucleus. See figure 33.3 for additional explanation, a' significantly ( $P < 0.05$ ) larger than a, Duncan's Multiple Range Test.



**Figure 33.5**

The influence of neonatal hormonal treatment on the morphological sex difference within the medial preoptic nucleus (MPON) and on the volume of the suprachiasmatic nucleus (SCN). Female rats were injected with 0.05 ml sesame oil on day 4, or with oil plus 90 µg testosterone propionate (TP), or 1 mg TP, and ovariectomized two weeks prior to sacrifice. Males were orchidectomized on day 1 or 21. Numbers of animals are indicated at the bottom of each bar which represents the mean volume  $\pm$  S.E. †Significantly ( $P < 0.01$ ) larger than any other group; \*\*Significantly ( $P < 0.01$ ) larger than oil-female; \*Significantly ( $P < 0.05$ ) larger than oil-female. Duncan's Multiple Range Test.

5 (males shamcastrated on day 1) were significantly ( $P < 0.01$ ) greater than any other group. Moreover, the neonatally castrated males (Group 4) and the females androgenized with 1 mg testosterone propionate (Group 3) were significantly larger than the nuclear volume of the control female group. With respect to the volume of the SCN, one-way ANOVA revealed that there was no significant difference. As illustrated in table 33.2, POA height, POA width and septal width did not vary with treatment. Although there was a significant difference in brain weight, the sexual dimorphism in the MPON persisted when the volume was expressed relative to brain weight. Figure 33.6 presents representative sections through the MPON in the 4 groups of animals which exhibited significant differences.

## Discussion

The major findings of the present study are that there is a gross morphological sexual dimorphism in the MPON of the rat, a sexual dimorphism which is independent of the hormone environment of the adult, but one that is at least partially determined by the perinatal hormone environment. The fact that volume of the MPON of the neonatally castrated male is not equivalent to that of the female, or that this volume in the androgenized female fails to reach that of the male castrated at weaning, suggests that partial differentiation may occur prenatally, and/or that the volume of the

MPON is influenced by the neuronal genome. In an attempt to elucidate this question, the development of this sexual dimorphism is currently under investigation. In addition, preliminary quantitative analysis of thin sections prepared from the same area suggests that this sexual dimorphism is due to an increase in the number of relatively large (10–15 µm in diameter) cells, presumably neurons, in males rather than to a sex difference in regional cell packing. Although staining characteristics of these cells may underlie the sex difference, this dimorphism is also present in cresylecht violet stained sections (unpublished observations).

In addition to the marked sex difference in MPON volume, the present results also suggest the possibility that the SCN nucleus may also be morphologically sexually dimorphic, and as well that the volume of both nuclei may be influenced by the hormone environment in the adults. Although there is some precedent for such changes, since hormones have been shown to alter neuronal nuclear and nucleolar volume (5, 6, 16, 22), the present results must be considered preliminary only. Although the present analyses were performed without knowledge of treatment group, delineation of the intensely staining component of the MPON has an element of subjectivity. To evaluate the reliability of this method, the nuclear volume of 8 regions was estimated a second time, again without knowledge of treatment group or previous data. Statistical analysis of the original and repeat estimates of nuclear volume revealed a variance of  $15.0 \pm 4\%$ , without respect to

**Table 33.2**

The influence of the neonatal hormone environment on brain size and volume of the medial preoptic nucleus (MPON) and suprachiasmatic nucleus (SCN) relative to brain size in adult gonadectomized rats

Group	Treatment	Number of Rats	Preoptic Height (mm)	Preoptic Width (mm)	Septal Width (mm)	Brain Wt. (g)	MPON Vol. Brain Wt. (cu. mm/g)	SCN Vol. Brain Wt. (cu. mm/g)
<i>Female</i>								
1	Oil-day 4	3	1.85 ± 0.05	1.25 ± 0.03	3.05 ± 0.15	1.82 ± 0.02	0.08 ± 0.01	1.19 ± 0.01
2	90 µg TP day 4	5	1.81 ± 0.04*	1.30 ± 0.06*	3.01 ± 0.07	1.79 ± 0.04	0.14 ± 0.02	1.04 ± 0.07*
3	1 mg TP day 4	4	1.91 ± 0.05	1.27 ± 0.12	3.14 ± 0.06	1.76 ± 0.01	0.21 ± 0.05 <sup>††</sup>	1.17 ± 0.05
<i>Male</i>								
4	Gonadx on day 1	6	1.93 ± 0.04**	1.31 ± 0.06**	3.18 ± 0.03	1.77 ± 0.04	0.25 ± 0.03 <sup>†††</sup>	1.07 ± 0.10
5	Gonadx on day 21	5	1.90 ± 0.04	1.39 ± 0.03	3.23 ± 0.04	1.93 ± 0.03 <sup>***</sup>	0.45 ± 0.03 <sup>†</sup>	1.09 ± 0.02

Abbreviations: TP, testosterone propionate; Gonadx, gonadectomized.

\*One less animal in this group.

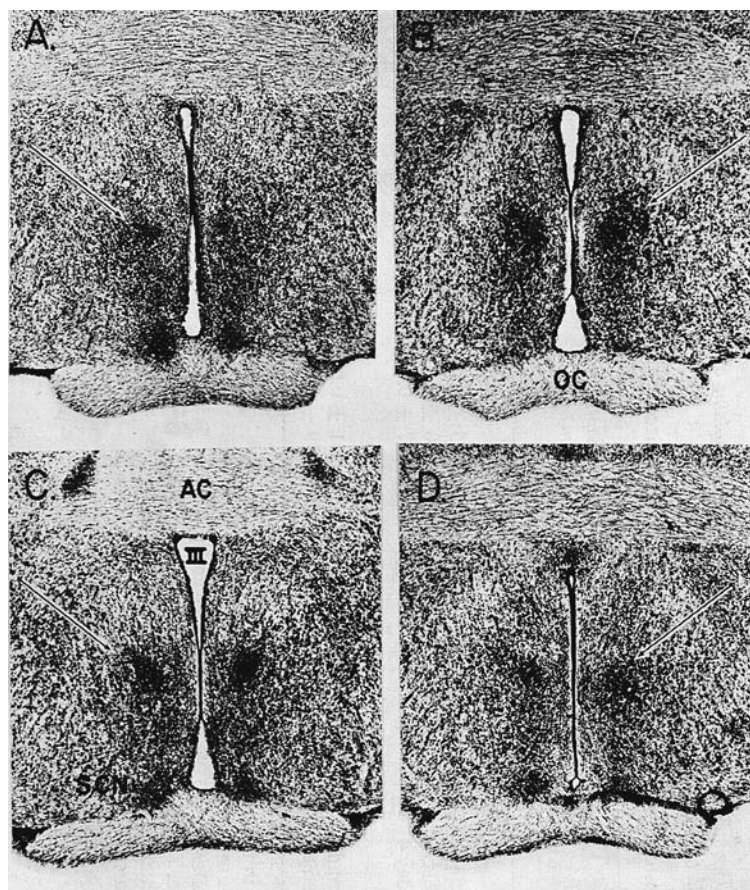
\*\*Two less animals in this group.

\*\*\*Significantly greater ( $P < 0.05$ ) than any other group.

<sup>†</sup>Significant greater ( $P < 0.01$ ) than any other group.

<sup>††</sup>Significantly greater ( $P < 0.05$ ) than Group 1.

<sup>†††</sup>Significantly greater ( $P < 0.01$ ) than Group 1.

**Figure 33.6**

Representative coronal sections through the medial preoptic nucleus (arrows) of the gonadectomized female (A), male (B), androgenized female (C; 1 mg testosterone propionate) and neonatally castrated male (D). These animals were chosen for illustration because their individual nuclear volumes most closely approximated their group means; Thionine. All at same magnification. Abbreviations as in figure 33.2.

sign. It is possible, therefore, that the minor and apparently hormone-dependent changes in nuclear volume, although statistically significant, are within the error of the assay system. Nevertheless, it should be emphasized that hormone-dependent subtle morphological differences in the adult brain may well exist independently of the gross sexual dimorphism.

Although the marked difference in volume of the MPON is clearly and highly statistically significant, the question of the biological meaning of this observation is far from certain. Although it is tempting to consider that the morphological differences in the MPON may be a reflection of a fundamental sexually dimorphic function that may be served by these cells, it must be pointed out that the identity of these cells as neurons is not firmly established. However, it is likely that we have measured a sex difference in neuronal organization. With respect to specific sexually dimorphic functions which might be expected to correlate with this morphological difference in absolute volume, it would appear that the regulation of the cyclic discharge of GTH or of lordosis behavior are not likely candidates. Although the influence of the perinatal hormone environment on nuclear volume is clearly evident in figure 33.5, note that the oil-treated female and neonatally castrated male are the only two animal preparations among these 5 which display the potential for cyclic GTH release or display high levels of lordosis behavior, yet their nuclear volumes are significantly different. Moreover, there is no significant difference in nuclear volume between androgenized females and the neonatally castrated male, yet their functional potential, with respect to GTH secretion or lordosis behavior, differs markedly.

In contrast, the nuclear volumes illustrated in figure 33.5 are relatively consistent with the level of masculine behavior exhibited by these preparations (21, 28, 30, 31), and several studies suggest that the POA is important for the display of masculine sexual behavior (see refs. 2, 3, 10, 15). However, the preceding studies were not conducted with knowledge of the existence of the morphological sexual difference within the MPON. Therefore, it would be premature to assign this nucleus a specific role in the regulation of masculine sexual behavior to the exclusion of any other of the many functions of the hypothalamus. Note that it is not actually known whether the neurons of the MPON of the male are facilitatory or inhibitory to any particular function. The fact that this sexual dimorphism is readily identified, and restricted to a relatively discrete and consistent location, does present a unique opportunity to evaluate the possible mechanisms of hormone induced sexual differentiation of the brain, and perhaps neuronal differentiation in general.

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The literature of the past century on the possibility of morphological sex differences in the human brain is a mixture of scientific observations and cultural bias, in which male and female “superiority” were alternately contended (1, 2). Until recently, however, Mall’s conclusion that “each claim for specific [sex] differences fails when carefully tested” (3, p. 27) held true with respect to the human brain, apart from the sex difference in overall size of the brain (1). A few years ago a sex difference in the shape of the corpus callosum was described (4), and recently the shape of the suprachiasmatic nucleus (SCN) was found to be sexually dimorphic (1). Yet, to our knowledge, no sex difference in cell number has been reported for any human brain area. On the other hand, since Raisman and Field (5) reported sex differences in the synaptic organization of the preoptic area in the rat, reports pertaining to gender-linked differences in many brain components throughout the animal kingdom have increased (1). The most conspicuous of these sex differences was described by Gorski et al. within the rat brain (6), in the preoptic area (POA). A cell group within this area revealed such a clear cytoarchitectonic sex difference that it could even be seen with the naked eye in Nissl-stained sections. We have studied an analog of this sexually dimorphic nucleus (SDN) of the POA in the human brain. Morphometric analysis demonstrates that the SDNPOA is  $2.5 \pm 0.6$  times [mean  $\pm$  standard error of the mean (S.E.M.)] as large in men as in women and contains  $2.2 \pm 0.5$  times as many cells.

Brains of 13 men and 18 women between 10 and 93 years of age were fixed, generally for 1 month, in Formalin. Serial coronal sections (6  $\mu$ m) were taken from the hypothalamus and stained with thionin (7).

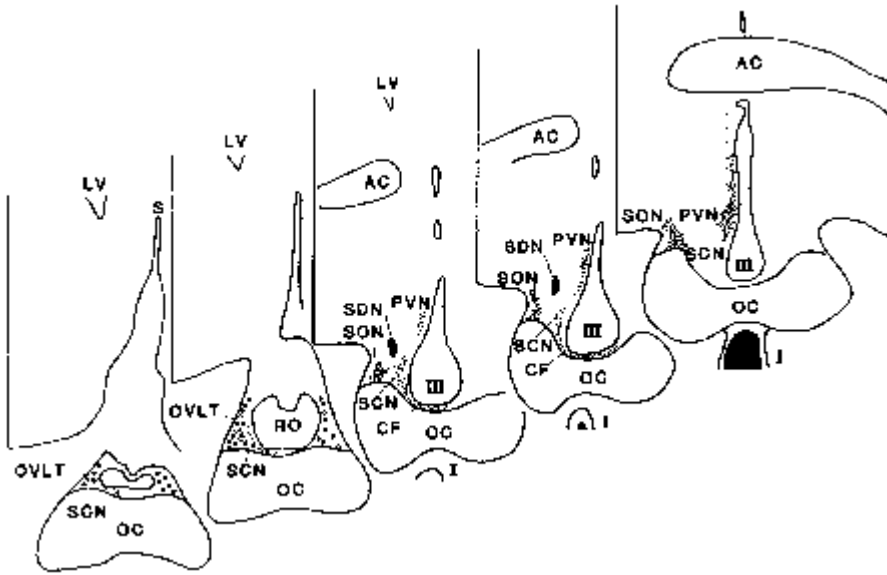
As has been reported for the rat (6), the SDN-POA was characterized by its more intense staining, larger cell bodies, and higher cell density than the rest of the POA. The SDN-POA was located in the medial POA, between the dorsolateral supraoptic nucleus and the rostral pole of the paraventricular nucleus (figures 34.1 and 34.2). It was generally present in the same sections that contained the suprachiasmatic nucleus, which had been marked by antivasopressin (2, 8).

The rostrocaudal axis of the SDN-POA and its maximum cross-sectional surface were measured to describe the shape and to calculate SDN-POA volume (9). In addition, cell density was measured (10), which, in combination with SDN-POA volume, allowed for calculation of total SDN-POA cell numbers. The SDN-POA measurements were performed on the same side of the brain and in the same subjects as the earlier suprachiasmatic nucleus measurements (1, 8).

The SDN-POA volumes were, respectively,  $2.2 \pm 0.6$ ,  $2.0 \pm 0.6$ , and  $3.3 \pm 1.3$  times as large in men as in women in the three age groups (table 34.1) (11, 12). Total cell number was, respectively,  $1.74 \pm 0.36$ ,  $1.96 \pm 0.62$ , and  $2.75 \pm 0.79$  times as large in men as in women (figures 34.2 and 34.3) (11). Also when SDN-POA volume was expressed as a ratio to brain weight, the values were significantly larger in men ( $131.3 \pm 18.8 \times 10^{-6}$  mm<sup>3</sup>/g) than in women ( $63.5 \pm 9.8 \times 10^{-6}$  mm<sup>3</sup>/g) (11). The maximum cross-sectional area through the SDN-POA was  $2.1 \pm 0.4$  times as large in men as in women (11). The SDN-POA attained its maximum cross-sectional area 300  $\mu$ m caudal to the section containing the maximum area of the SCN in women and 500  $\mu$ m caudal in men. The values of the Alzheimer’s patients were commensurate with their ages and have therefore been included in the study.

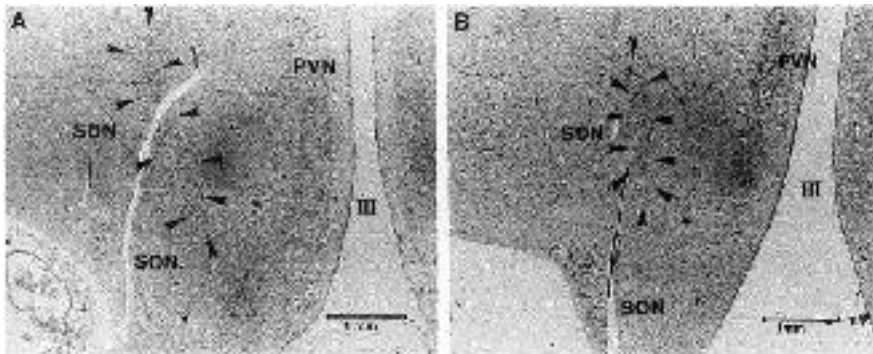
In both sexes SDN-POA volume (in men, to  $43 \pm 17$  percent; in women, to  $29 \pm 7$  percent), cell number (to  $46 \pm 13$  percent and  $29 \pm 6$  percent), and maximum cross-sectional area (to  $49 \pm 10$  percent and  $52 \pm 9$  percent) decreased with advancing age (figure 34.3 and table 34.1) (11).

Sex differences in the size of the SDN-POA in the rat are independent of sex hormonal treatment in adulthood (13). Our series included the brain of a 46-year-old woman who had been virilized by a tumor of the adrenal cortex (7). Her SDN-POA measurements were similar to the other female values, which is in agreement with Gorski’s data on the rat (13). In our series no information is available with respect to still another SDN-POA property described in the rat—the reversibility of sexual dimorphism by hormonal manipulation in earlier development.



**Figure 34.1**

(top left). Topography of the human hypothalamus. Abbreviations: AC, anterior commissure; CF, commissural fibers of the suprachiasmatic nucleus; I, infundibulum; LV, lateral ventricle; OC, optic chiasm; RO, recessus opticus; OVLT, organum vasculosum of the lamina terminalis; PVN, paraventricular nucleus; S, septum; SDN, sexually dimorphic nucleus of the POA; SCN, suprachiasmatic nucleus; SON, dorsolateral supra-optic nucleus; III, third ventricle.



**Figure 34.2**

(bottom left). Thionin-stained frontal sections (6  $\mu$ m) of the hypothalamus of (A) a 28-year-old man and (B) a 10-year-old girl. Arrows show the extent of the SDN.

The differences with respect to age and sex are unlike those observed for the SCN in the same brain material. In the latter area a sex difference was found only in shape (for example, in the length of the rostrocaudal axis) but not in volume or cell number. In addition, no decline in volume and cell number in the SCN was observed until after the age of 80 years (8). This result demonstrates that the sex and age differences reported for the human SDN-POA are not part of a general effect on the hypothalamus but are localized.

The preoptic area plays a role in gonadotropin release and sexual behavior in many species (14). Transplantation of the POA from newborn males to the same area in female littermates enhances masculine and feminine sexual behavior in adulthood (15). In the male

monkey, changes in neuronal activity in the medial POA are related to the initiation of sexual behavior, penile erection, and the refractory period following ejaculation (16).

In addition, neurons of the POA concentrate androgens and estrogens (17), a function presumed to be instrumental in the development of sex differences in this area. The function of the SDN-POA, on the other hand, is unknown both in the rat and in humans. Lesions restricted to this nucleus in the rat failed to reveal a role in male sexual behavior for this part of the POA (18). Sexually dimorphic areas have also been identified within the POA's of the gerbil, ferret, guinea pig, hamster, and mouse (19), but interspecific homologies of these sexually dimorphic areas remain to be

**Table 34.1**  
Individual data for men and women separately (N.D., not determined)

Age Group	Men						Women					
	Age (Years)	Body Height (cm)	Brain Weight (g)	SDN-POA		Cell Number ( $\times 10^{-5}$ )	Age (Years)	Body Height (cm)	Brain Weight (g)	SDN-POA		Cell Number ( $\times 10^{-5}$ )
				Volume (mm <sup>3</sup> )	Volume (mm <sup>3</sup> )					Volume (mm <sup>3</sup> )	Volume (mm <sup>3</sup> )	
10 to 40	16	180	1940	0.570		52.87	10	N.D.	1270	0.166		29.15
	19	191	1900	0.123		16.70	15	179	1480	0.129		18.06
	22	190	N.D.	0.233		44.54	30	165	1460	0.081		16.36
	27	180	1560	0.177		31.85	35	174	1200	0.137		25.50
	28	189	1510	0.280		38.12	38	154	1360	0.101		13.00
	31	173	1330	0.234		29.21						
$\bar{X} \pm S.E.M.$	$23.8 \pm 2.4$	$183.8 \pm 3.0$	$1648 \pm 118$	$0.270 \pm 0.064$		$35.55 \pm 5.15$	$25.6 \pm 5.6$	$168.0 \pm 5.5$	$1354 \pm 54$	$0.123 \pm 0.015$		$20.41 \pm 2.99$
41 to 70	43	176	1260	0.128		31.22	46	181	1360	0.142		24.63
	59	177	1350	0.201		17.96	52	173	1370	0.061		7.57
	61	177	1400	0.183		31.60	57	164	1220	0.072		14.36
							60	160	1110	0.161		27.17
							64	169	1090	0.019		2.36
							70	165	780	0.019		3.12
							70	N.D.	1210	0.120		17.20
$\bar{X} \pm S.E.M.$	$54.3 \pm 5.7$	$176.7 \pm 0.3$	$1337 \pm 41$	$0.171 \pm 0.022$		$26.93 \pm 4.48$	$59.9 \pm 3.4$	$168.7 \pm 3.1$	$1163 \pm 76$	$0.085 \pm 0.022$		$13.77 \pm 3.75$
71 to 100	74	172	1410	0.027		4.58	72	165	1200	0.007		9.21
	83	178	1280	0.082		18.87	88	160	1030	0.033		5.94
	85	165	1400	0.203		23.26	90	160	1110	0.058		7.31
	87	N.D.	1275	0.148		18.53	90	N.D.	1300	0.057		4.49
							91	160	1060	0.033		4.96
							93	165	1020	0.022		3.67
$\bar{X} \pm S.E.M.$	$82.3 \pm 2.9$	$171.7 \pm 3.8$	$1341 \pm 37$	$0.115 \pm 0.038$		$16.31 \pm 4.06$	$87.3 \pm 3.1$	$162.0 \pm 1.2$	$1120 \pm 45$	$0.035 \pm 0.008$		$5.93 \pm 0.83$

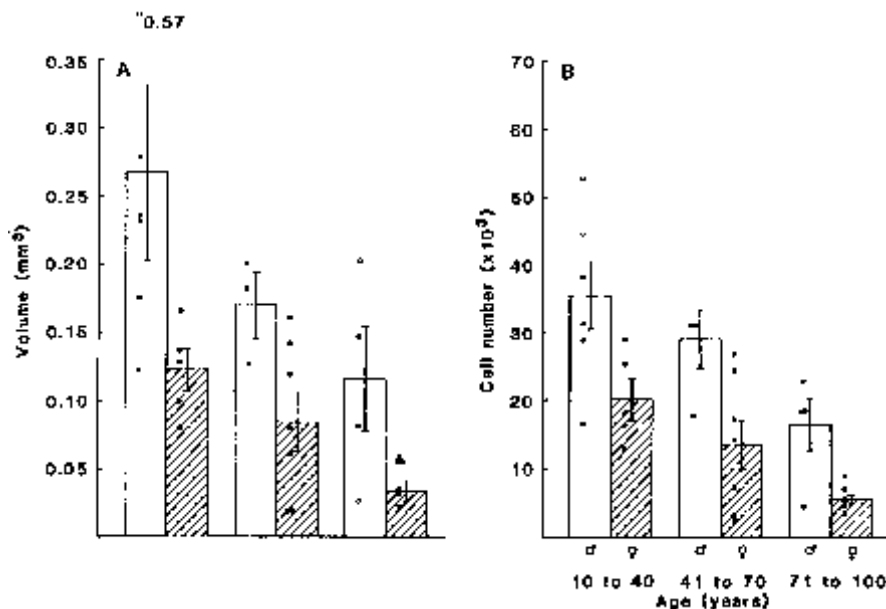


Figure 34.3

(right). (A) Volume and (B) cell number of the human SDN-POA (means and S.E.M.'s). Points represent individual values.

shown. Immunocytochemistry might be of value for this purpose, since in the rat intense innervation by cholecystokinin-containing fibers, a lack of innervation by serotonin fibers, and a sex difference for neurotensin and substance P cell bodies have been reported (20). In addition, immunocytochemistry seems to be a potent technique for studying the exact chemical nature of the SDN-POA sex differences and the cell types in which the changes with aging occur.

#### References and Notes

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7. Brains of 31 patients (13 men and 18 women) were obtained at autopsy. Subjects had no neurological disease, except for one man and two women who had been diagnosed clinically and pathologically as suffering from senile dementia of the Alzheimer's type. One 46-year-old woman had suffered during at least 1 year from a tumor of the adrenal cortex, which induced high concentrations of androstenedione and testosterone in the blood. At autopsy, male brains weighed  $1467.9 \pm 66.3$  g (mean  $\pm$  standard error of the mean) and female brains weighed  $1201.7 \pm 41.5$  g (18.1 percent smaller) (table 34.1). Brains were fixed in 10 percent formaldehyde at room temperature generally for 30 days. The hypothalamic area was subsequently dissected, dehydrated, and embedded in paraffin. Serial 6- $\mu$ m frontal sections were cut on a Leitz microtome, mounted on chromium aluminum sulfate-coated slides, hydrated, brought to phosphate-buffered saline, and stained with thionin.
8. D. F. Swaab et al., in *Topics in Aging Research*, D. L. Knook et al., Eds. (Eurage, Brussels, 1984), vol. 2, pp. 71–78.
9. The rostrocaudal length of the SDN-POA was determined by staining every 25th section from the lamina terminalis to the caudal end of the optic chiasm with thionin. The rostral and caudal borders of the SDN-POA were assessed by subsequent staining of every fifth section in the most rostral and the most caudal parts and by determining the sections in which the first and last SDN-POA cells were present. Area measurements of the cross-sectional SDN-POA and the cell nuclei were performed by means of a Calcomp digitizer, through the use of a Zeiss microscope with  $\times 10$  and  $\times 40$  (plan) objectives, respectively, and  $\times 12.5$  plan oculars. The maximum cross-sectional SDN-POA area is presented as a separate measure. The volume of the SDN-POA was determined by integrating area measurements from the first to the last SDN sections [C. G. Van Eden et al., *Dev. Brain Res.* 12, 146 (1984)], 11  $\pm$  3 sections (mean  $\pm$  standard error of the mean) being measured per subject.
10. The number of SDN cells per (unit) volume (cell density) was estimated through the use of a discrete "unfolding" procedure [E. R. Weibel, *Stereological Methods*, vol. 1, *Practical Methods for Biological Morphometry* (Academic Press, New York, 1979)], which included the modification for classification proposed by L. M. Cruz-Orive [*J. Microsc.* 112, 153 (1978)] and a correction for section thickness (6  $\mu$ m). The nuclear profiles of  $132 \pm 6$  cells (mean  $\pm$  standard error of the mean) per SDN-POA were measured per subject for this procedure in the section containing the maximum SDN-POA area. The computer program for these procedures was developed in our institute by R. W. H. Verwer.
11. The influence of age and sex on SDN-POA length, maximum area, cell density, and cell number were tested by means of two-way analysis of variance ( $\alpha = 0.05$ ). Significant sex (main) effects were found for maximum area [ $F(1, 25) = 18.21$ ;  $P < 0.001$ ], volume [ $F(1, 25) = 12.17$ ;  $P = 0.002$ ], cell number [ $F(1, 25) = 15.97$ ;  $P = 0.001$ ], and the ratio of SDN-POA volume to brain weight [ $F(1, 25) = 11.63$ ;  $P = 0.002$ ]. Significant age (main) effects were found for the decline in maximum area [ $F(2, 25) = 6.61$ ;  $P = 0.005$ ], volume [ $F(2, 25) = 5.52$ ;  $P = 0.001$ ], cell number [ $F(2, 25) = 9.44$ ;  $P = 0.001$ ], and the ratio of SDN-POA volume to brain weight [ $F(1, 25) = 5.29$ ;  $P = 0.012$ ], while no significant interactions between the effects of age and sex on these variables were found.

( $P > 0.25$ ). As in the rat (12), there was no statistically significant sex difference either in the length of the rostrocaudal axis ( $P = 0.162$ ) or in cell density ( $P = 0.937$ ) of the SDN-POA. In addition, no significant effects of postmortem delay ( $P > 0.05$ ) or duration of fixation ( $P > 0.05$ ) were found.

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21. We thank B. Fisser, P. J. van Nieuwkoop, G. v.d. Meulen, and H. Stoffels for their assistance, and F. C. Stam, W. Kamphorst, J. Jöbsis, J. M. Wigboldus, and H. Bakker-Winnubst for supplying the brain material. Supported by the Foundation for Medical Research (FUNGO; grant 13-51-30).



Male and female mammals exhibit differences in reproductive physiology and behavior patterns that are primarily controlled by the effects of gonadal hormones upon the brain (Goy and McEwen, 1980; Gorski, 1984). Several types of neuroanatomical sex differences have been identified in both mammalian and avian species that could underlie these functional differences. There are subtle sex differences in the size of nerve cell nuclei (Pfaff, 1966; Dörner and Staudt, 1968). At the ultrastructural level, several sexual dimorphisms have been identified in dendritic branching patterns of neurons of the preoptic area (POA) in the rat (Meyers and Gordon, 1982), hamster (Greenough et al., 1977), and the macaque monkey (Ayoub et al., 1983); and in the synaptic organization of the POA (Raisman and Field, 1971), arcuate nucleus (Matsumoto and Arai, 1981), and medial amygdala (Nishizuka and Arai, 1981) of the rat. In addition to these subtle microscopic differences, there exist dramatic dimorphisms in cell groups involved in vocal communication in canaries and finches (Nottebohm and Arnold, 1976); in the bed nucleus of the stria terminalis (BNST) in guinea pigs (Hines et al., 1985) and in rats (del Abril et al., 1987); and in the POA of rats (Gorski et al., 1978, 1980), gerbils (Yahr and Commins, 1982), guinea pigs (Hines et al., 1985), ferrets (Tobet et al., 1986), and quail (Panzica et al., 1987).

Despite many reports of sexually dimorphic structures in mammalian and avian species, relatively little is known about neuroanatomical sex differences in the human brain. There are gender-related allometric variations in brain weight and evidence for sexual dimorphism in morphological brain asymmetry (Wada et al., 1975). In addition, the massa intermedia (MI) is more often present (Rabl, 1958), and both the MI (Allen and Gorski, 1987) and the anterior commissure (Allen and Gorski, 1986) are larger at the midsagittal plane in women than in men. Similarly, sex differences in the corpus callosum have been reported (de LaCoste-Utamsing and Holloway, 1982), although not all subsequent studies have replicated this finding. However, there remains a dearth of documented volumetrically

sexually dimorphic cell groups in the human brain (Swaab and Fliers, 1985).

Because the POA shows the greatest number of reported gender-related dimorphisms in other mammalian species, it is a likely site for similar differences in humans. Furthermore, the POA has been implicated in several reproductive functions in the rat and subhuman primate such as gonadotropin release (Gorski, 1968; Plant et al., 1979; Pohl and Knobil, 1982; Wiegand and Terasawa, 1982), maternal behavior (Jacobson et al., 1980), and sexual behavior (Robinson and Mishkin, 1966; Gorski, 1974; Arendash and Gorski, 1983; Oomura et al., 1983). Since there are no clear boundaries in the human brain between the POA and the anterior hypothalamus, and, in fact, some anatomists consider the POA to be the anterior region of the anterior hypothalamus, we selected the preoptic-anterior hypothalamic area (PO-AHA) for quantitative analysis of possible sexually dimorphic nuclei in the human brain.

#### Materials and Methods

The brains of 22 human subjects, which were determined to be normal upon autopsy and were from individuals who had no known neurological disorder, were chosen from our brain bank of about 100 hypothalami to represent a chronological range of age-matched male and female individuals. All of these brains were collected from 1 of 2 Southern California hospitals, removed from their skulls within 8 hr post-mortem, and placed directly in acetate-buffered 10% formalin (ABF) for 2–4 weeks prior to coronal sectioning performed during routine autopsy. Hypothalami were dissected from these coronal slabs, and these samples, approximately 3 cm<sup>3</sup>, were saved for 3 months to 2 years prior to histological preparation. No changes due to duration of fixation were observed when comparing the volume of nuclei from brains that had been fixed for less or more than a year (ANOVA;  $p > 0.05$ ). Subsequently, all samples were processed for histology in age-matched pairs as described below.

**Table 35.1**

Individual data on the interstitial nuclei of the anterior hypothalamus (INAH), SON, cause of death, and race

Pair	Age (yr)	Brain Weight (gm)	INAH-1 Volume (mm <sup>3</sup> )	INAH-2 Volume (mm <sup>3</sup> )	INAH-3 Volume (mm <sup>3</sup> )	INAH-4 Volume (mm <sup>3</sup> )	SON Volume (mm <sup>3</sup> )	Cause of Death	Race
<i>Males</i>									
1	5	1210	0.424	0.046	0.095	0.049	6.552	Lymphoblastic leukemia	Hispanic
2	29	1460	0.404	0.049	0.078	0.149	6.570	Lymphoblastic leukemia	White
3	29	1270	0.465	0.037	0.130	0.004	9.569	Burkitt's lymphoma	White
4	31	1490	0.452	0.048	0.103	0.205	9.252	Chronic myelogenous leukemia	White
5	43	1400	0.343	0.100	0.156	0.045	8.751	Neuroadenoma	Hispanic
6	58	1450	0.321	0.046	0.132	0.150	6.199	Squamous cell carcinoma	White
7	58	1195	0.271	0.046	0.138	0.082	5.804	Heart disease	White
8	63	1250	0.473	0.030	0.315	0.077	7.583	Heart disease	White
9	68	1240	0.366	0.026	0.113	0.070	6.010	Heart disease	White
10	72	1420	0.241	0.029	0.126	0.038	7.285	Heart disease	White
11	81	1380	0.267	0.030	0.068	0.073	4.259	Adenocarcinoma	White
Mean	48.8	1342.3	0.366	0.044	0.132	0.086	7.076		
SEM	6.66	31.68	0.024	0.006	0.019	0.017	0.487		
<i>Females</i>									
1	4	1120	0.512	0.005	0.005	0.003	5.435	Heart disease	White
2	20	1350	0.431	0.041	0.085	0.127	6.514	Burkitt's lymphoma	White
3	25	1150	0.421	0.050	0.008	0.078	4.937	Aplastic anemia	White
4	32	1200	0.218	0.047	0.062	0.102	7.511	Budd-chiari syndrome	White
5	44	1275	0.240	0.011	0.034	0.016	6.862	Sickle cell disease	Black
6	61	1280	0.211	0.014	0.029	0.048	4.843	Adenocarcinoma	White
7	63	1090	0.310	0.009	0.006	0.044	7.226	Heart disease	White
8	64	1190	0.248	0.010	0.102	0.018	6.306	Cryptogenic cirrhosis	White
9	69	1280	0.234	0.009	0.059	0.096	6.196	Heart disease	White
10	72	1250	0.244	0.027	0.081	0.066	5.560	Heart disease	White
11	81	1260	0.216	0.015	0.049	0.017	4.677	Diabetes mellitus	White
Mean	48.6	1222.3	0.299	0.022	0.047	0.056	6.007		
SEM	7.200	22.758	0.030	0.005	0.010	0.012	0.296		

**Immersion in Gelatin**

Blocks were rinsed overnight in cool water to remove ABF, immersed in 5% gelatin at 37°C for 24 hr, immersed in 10% gelatin for 24 hr, embedded in 10% gelatin, which was allowed to solidify at room temperature, trimmed of excess mold to expose the brain, and placed in 10% ABF for 4 d to harden.

**Sectioning**

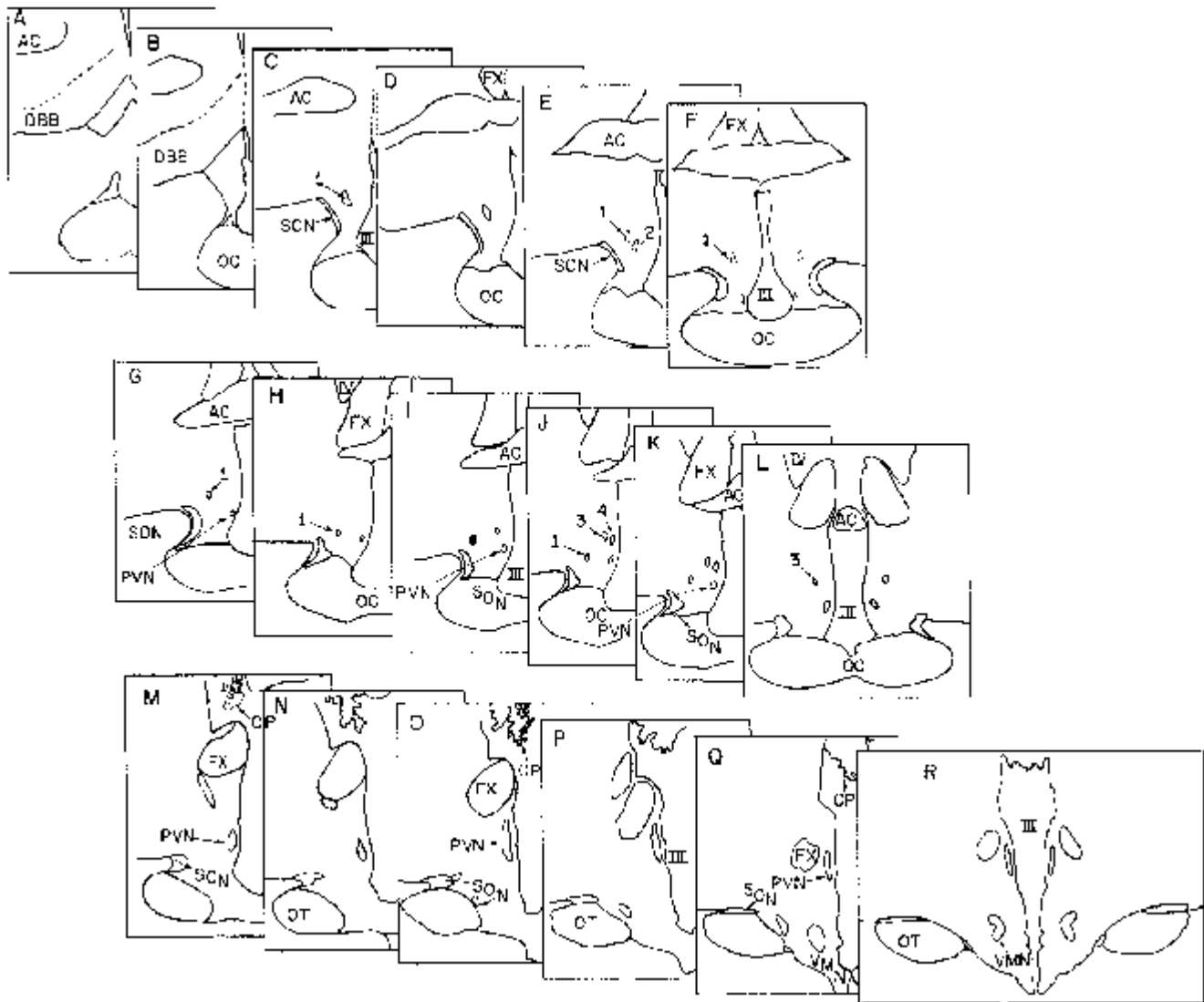
Serial 60  $\mu$ m coronal sections were mounted from saline onto gelatin-coated glass slides, and incubated at 37°C overnight.

**Staining with Thionin**

Sections were dehydrated with alcohol, defatted with xylene, rehydrated with distilled water, stained with 1% buffered thionin, rinsed with distilled water, differentiated with ABF followed by alcohol, and coverslipped out of xylene.

Since we were unable to identify any cell group clearly homologous to a sexually dimorphic nucleus of another species, 4 relatively discrete cell groups within the PO-AHA that stained darkly with thionin were selected for quantitative analysis. In our search of the

literature on the human hypothalamus, we were unable to determine the nomenclature for any of these 4 cell groups. We therefore elected to name them Interstitial Nuclei of the Anterior Hypothalamus (INAH) in numerical order in a lateral to medial direction. In addition, we selected the supraoptic nucleus (SON) as a control nucleus since it has not been reported to be sexually dimorphic in another species, is located in the region of the INAH, and, like the INAH, has well-defined borders, relative to the paraventricular nucleus (PVN), the borders of which are not well-defined in the thionin-stained human brain. Serial sections of the INAH and SON were projected onto paper at  $\times 44$  magnification, and the boundaries were traced independently by 3 investigators who had no knowledge as to the sex of the brains. We arbitrarily chose to draw the nuclei located on the right side of the brain. The 3 tracings were superimposed on a light box using other anatomical landmarks, and an average outline common to a minimum of 2 of the individual tracings was drawn for each section of each cell group. In general, there was close agreement among the 3 investigators, and the composite outline approximated an average of all 3 individual drawings. We use this methodology

**Figure 35.1**

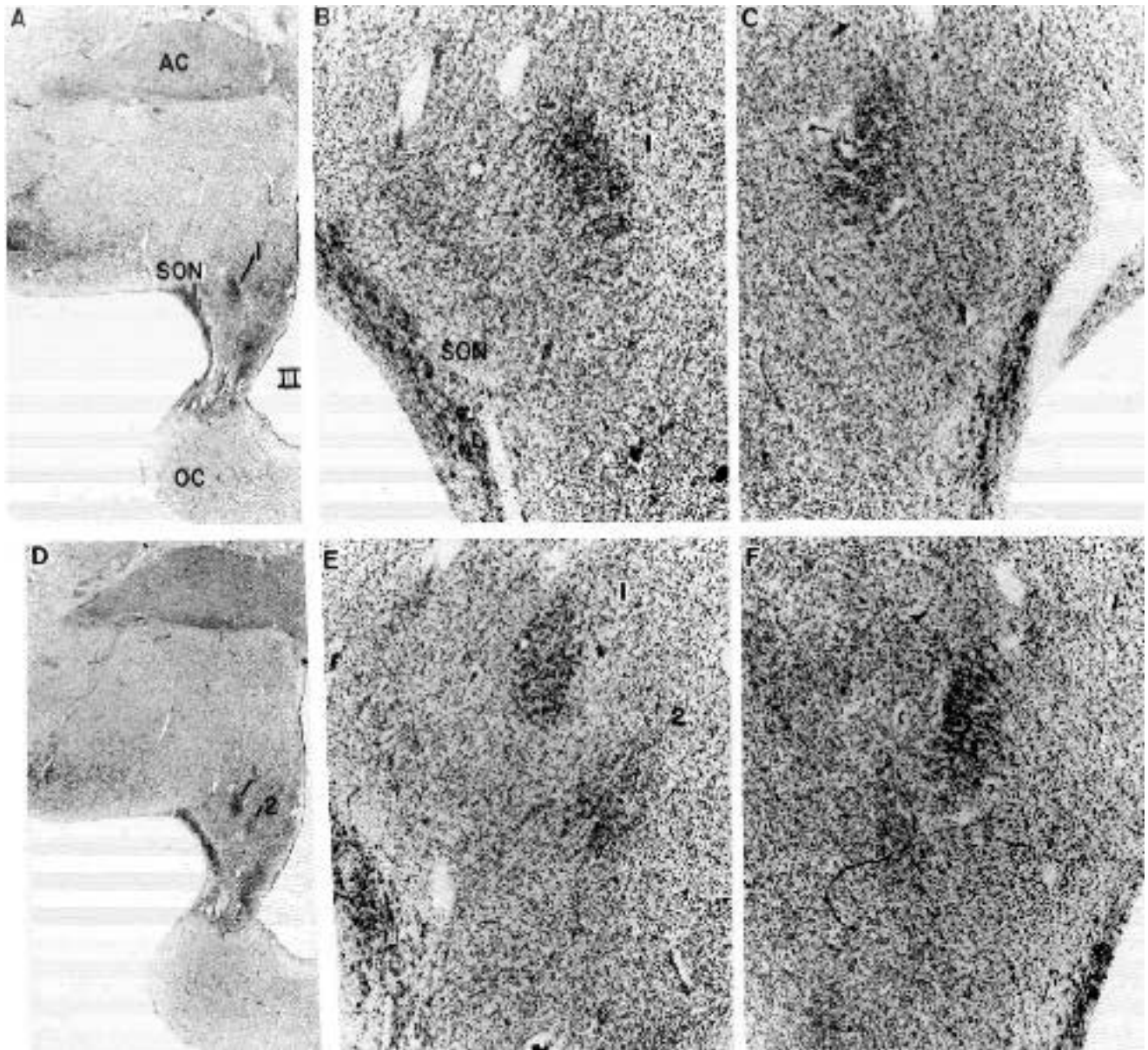
Schematic illustration of the nuclei analyzed in this study in the coronal plane, organized rostrally to caudally, from the diagonal band of Broca (DBB) to the ventromedial nucleus (VMN). This atlas was drawn from sections, all projected at the same magnification, from the 58-year-old male from pair number 7 who was selected because of the similarity in the plane of section to his age-matched pair (table 35.1). There are 7 60- $\mu$ m-thick sections between each section from A through C and from L through R. Between each section from C through L, which contain the INAH, there are only 2 60- $\mu$ m-thick sections. Levels D, E, I, and J correspond to the figures 35.2A, 2D, 2G, and 2J, respectively. Other abbreviations: anterior commissure, AC; optic chiasm, OC; INAH-1, 1; fornix, FX; third ventricle, III; INAH-2, 2; supraoptic nucleus, SON; paraventricular nucleus, PVN; INAH-3, 3; INAH-4, 4; lateral ventricle, LV; choroid plexus, CP; and optic tract, OT.

rather than calculating an arithmetic mean of the 3 drawings because it more accurately reflects actual perceptions. For instance, in the case where 2 individuals traced a particular section, and a third individual did not, our composite drawing would represent an average drawing of the 2 investigators in agreement, rather than a smaller average based on all 3 investigators, but markedly different from the actual perception of any of the 3 individuals. This method of quantification has been employed routinely in evaluating the volume of neural structures in our laboratory (Gorski et al., 1978, 1980; Hines et al., 1985). The area of each average out-

line was determined using a Bioquant Hipad digitizer (Bioquant IBM Program version 2.1; R & M Biometrics) having a resolution of 0.5 mm. The volumes of these nuclei were calculated by summing the areas of these values, multiplying by the thickness of the sections, and correcting for magnification.

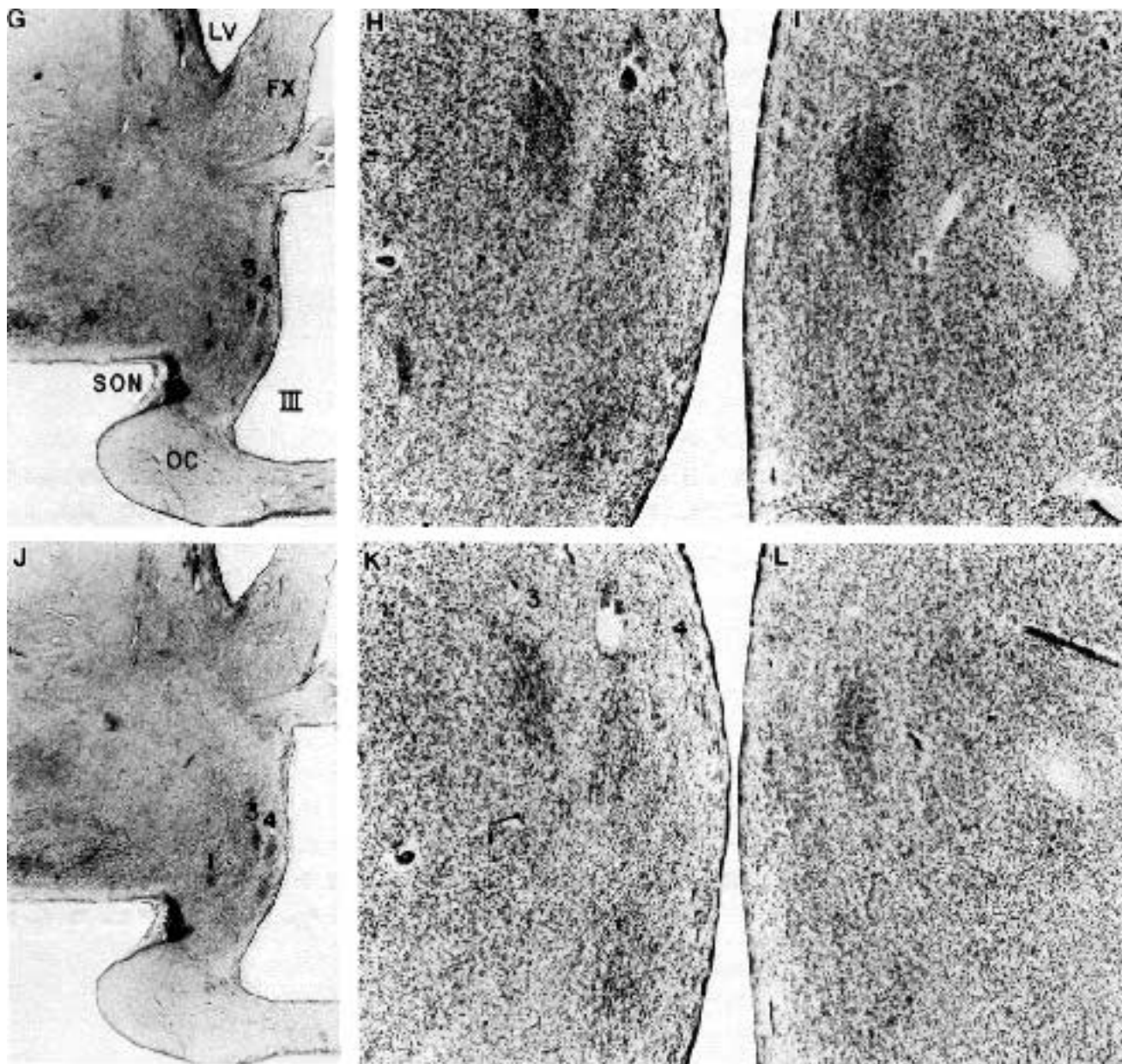
## Results

Our results are based on an analysis of the brains of 11 age-matched pairs of males and females. The age range for male subjects was 5–81 years (mean



**Figure 35.2**

*A–F*, Photomicrographs of thionin-stained coronal sections of the anterior hypothalamus of the human brain. Photographs *A* and *D* correspond to the schematics in figures 35.1*D* and 35.1*E*, respectively. *B*, Higher-power view of *A* showing greater detail of the supraoptic nucleus (SON) and INAH-1 (1). Similarly, *E* is a magnification of INAH-1 (1) and INAH-2 (2) from *D*. *C* and *F*, Sections at the same magnification and in the same region as *B* and *E*, respectively, from the 63-year-old female. Other abbreviations: anterior commissure, *AC*; optic chiasm, *OC*; and third ventricle, *III*. *G–L*, Photomicrographs of thionin-stained coronal sections, caudal to *A–F*, illustrating the interstitial nuclei of the anterior hypothalamus of the human brain. *G* and *J*, Sections corresponding to the figures 35.1*I* and 35.1*J*, respectively. *H*, Higher-power view of *J* showing greater detail of INAH-3 (3) and INAH-4 (4). Similarly, *K* is a higher-power view of *J*. *I* and *L*, sections at the same magnification and in the same region as *H* and *K*, respectively, from the 63-year-old female. Other abbreviations: fornix, *FX*; third ventricle, *III*; supraoptic nucleus, *SON*; optic chiasm, *OC*; lateral ventricle, *LV*; and INAH-1, *I*.



**Figure 35.2**  
(continued)

$\pm$  SEM =  $48.8 \pm 6.7$ ) and for female subjects was 4–81 years ( $48.6 \pm 7.2$ ). The average age difference between pairs was 2.3 years. There was an average of  $29.5 \pm 1.51$  sections per individual brain for INAH-1,  $5.09 \pm 0.26$  for INAH-2,  $9.7 \pm 1.01$  for INAH-3,  $7.95 \pm 0.97$  for INAH-4, and  $83.82 \pm 3.64$  for the SON. Although the brains were cut coronally during autopsy, the angle did vary slightly between subjects, and this increased the variability in the number of sections per nucleus between subjects since we could not always sacrifice hypothalamic tissue to adjust for plane of sectioning. Data were first analyzed using a paired *t*

test comparing the absolute volumes of each of the 4 cell groups for men and women (INAH-1,  $p > 0.05$ ; INAH-2,  $p < 0.03$ ; INAH-3,  $p < 0.002$ ; INAH-4,  $p > 0.05$ ; SON,  $p > 0.05$ ). Subsequently, we adjusted the absolute volumes for statistically significant sex differences in brain weight [average brain weight (in gm)  $\pm$  SEM for males was  $1342.3 \pm 31.7$ ; for females,  $1222.3 \pm 22.8$ ;  $p < 0.001$ ]. First, we divided the volume of each cell group by individual brain weight and analyzed these measures with the paired *t* test (INAH-1,  $p > 0.05$ ; INAH-2,  $p < 0.05$ ; INAH-3,  $p < 0.003$ ; INAH-4,  $p > 0.05$ ; SON,  $p > 0.05$ ). Sec-

ond, we performed an analysis of covariance of the absolute volumes of the nuclei by sex, with brain weight as the covariate (INAH-1,  $p > 0.05$ ; INAH-2,  $p < 0.02$ ; INAH-3,  $p < 0.001$ ; INAH-4,  $p > 0.05$ ; SON,  $p > 0.05$ ). With each of the 3 sets of statistics, INAH-2 and INAH-3 exhibited a statistically significant sexual dimorphism, while INAH-1, INAH-4, and the SON did not (table 35.1). Finally, the volume of each nucleus was adjusted with Durand's approximation (Burington, 1965), and all statistical results remained the same.

INAH-2, which is twice as large in males as in females, is located between the SON and the PVN (figures 35.1 and 35.2) and contains relatively small cell bodies (figure 35.3). Interestingly, INAH-2 was 3.7-fold larger in women of child-bearing age ( $n = 3$ ; 20–32 years old) than in the prepubescent ( $n = 1$ ) and postmenopausal females ( $n = 7$ ; the 44-year-old woman in this study had a hysterectomy with ovarian removal 3 years prior to death, and is therefore not included in the group of women of reproductive age). In fact, in the 3 women of reproductive age, INAH-2 was of similar volume to that of the males ( $0.046 \text{ mm}^3$  women vs.  $0.044 \text{ mm}^3$  in men). However, more data must be collected before valid statistical comparisons can be made between women of reproductive age and of nonreproductive age. None of the other 3 nuclei we quantified appeared to exhibit a similar age-related pattern of sexual dimorphism. The second sexually dimorphic nucleus that we identified, INAH-3, which was 2.8 times larger in males than in females regardless of age, is located slightly superior to the rostral pole of the PVN (figures 35.1 and 35.2) and has relatively large somata (figure 35.3).

The other 3 nuclei evaluated exhibited no statistically significant sexual dimorphism in volume. INAH-1, located dorsolateral to INAH-2, between the SON and the rostral pole of the PVN (figures 35.1 and 35.2), is a relatively large-celled nucleus (figure 35.3). In contrast to the other 3 INAH, which are somewhat spherical in shape, INAH-1 is tubular and oriented lengthwise along the rostrocaudal axis of the brain. Linear-regression analysis of the 5 nuclei in both males and females indicated that only the volume of INAH-1 significantly decreased with advancing age [ $p < 0.001$ ; correlation coefficient =  $-0.691$ ; slope of regression line ( $M$ ) =  $-0.0029$ ;  $Y$  intercept ( $B$ ) =  $0.475$ ; therefore, if  $X$  = average age  $\pm$  SEM,  $Y$  = average volume  $\pm$  SEM, and  $Y = B + MX$ , then  $0.332 \pm 0.021 = 0.4747 + (-0.0029)(48.727 \pm 4.9)$ ]. INAH-4 is also of similar size in both sexes. This nucleus, located superior to the rostral pole of the PVN and slightly posteromedial to INAH-3 (figures 35.1 and 35.2), contains relatively small cells (figure 35.3). The similarity in nuclear volume between the male and female INAH-1,

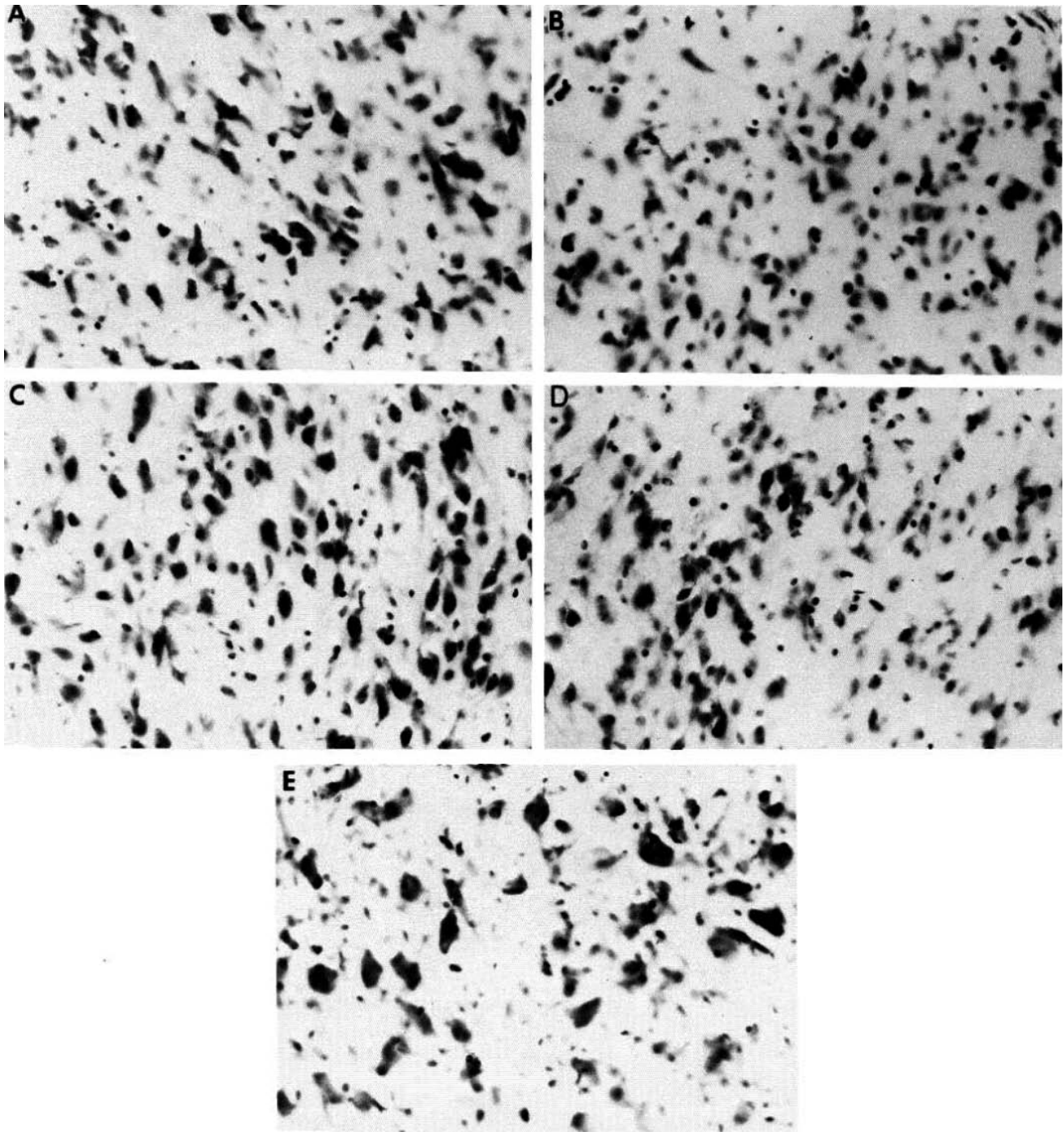
INAH-4, and the SON demonstrates that the sexual dimorphism observed in INAH-2 and INAH-3 does not reflect a general sexual dimorphism throughout the hypothalamus; rather, it is limited to certain structures.

## Discussion

### Homology of the INAHs to Nuclei of Other Species

It is unclear which, if either, of the 2 nuclei we found to be sexually dimorphic in the human brain corresponds to the SDN-POA of the rat. INAH-3 exhibits similarities to the SDN-POA of the rat by virtue of its location between the optic chiasm and the anterior commissure (figures 35.1 and 35.2) and its relatively large cell somata (figure 35.3). However, without knowledge of connectivity or neurochemical characteristics of these nuclei, it is difficult to assign any as a homolog to a sexually dimorphic nucleus of another mammalian species. In the rat, the medial preoptic nucleus (MPN), which contains the SDN-POA, receives input from the amygdala, ventral subiculum, ventral lateral septal nuclei, bed nucleus of the stria terminalis, insular cortical areas, nucleus accumbens, substantia innominata, raphe nuclei, ventral tegmental area, periaqueductal gray, pedunculopontine nucleus, and peripeduncular nucleus (Simerly and Swanson, 1986) and projects to the ventral tegmental area, midbrain central gray (Fahrbach et al., 1986), pedunculopontine nucleus, and zona incerta (Swanson et al., 1987). The MPN has been characterized immunohistochemically for the sexually dimorphic distribution of 5-HT fibers (Simerly et al., 1984), as well as for the presence of dopamine B-hydroxylase, neuropeptide Y, cholecystokinin, substance P, neurotensin, corticotropin-releasing factor, somatostatin, adrenocorticotrophic hormone, alpha-melanocyst stimulating hormone, leucine-enkephalin, and calcitonin gene-related peptide immunoreactive fibers; and substance P, leucine-enkephalin, cholecystokinin, thyrotropin-releasing hormone, neurotensin, corticotropin-releasing factor, calcitonin gene-related peptide, and vasoactive intestinal polypeptide-containing cell bodies (Simerly et al., 1986). Sex differences in the medial preoptic area (MPOA) have been identified in receptors for opiates (Hammer, 1984), 5-HT (Fischette et al., 1983), and gonadal steroids in the rat (Rainbow et al., 1982; Jacobson et al., 1987) and gerbil (Commins and Yahr, 1985); for acetylcholinesterase activity in the gerbil (Commins and Yahr, 1984); and for both acetylcholinesterase and choline acetyltransferase in the rat (Luine and McEwen, 1983).

Recently, considerable advances have been made in the localization of neurochemicals and receptors in the human brain, utilizing immunohistochemistry (Bouras et al., 1986) autoradiography (Palacios et al., 1986),



**Figure 35.3**  
Photomicrographs of 60- $\mu$ m-thick, thionin-stained sections from INAH-1 (*A*), INAH-2 (*B*), INAH-3 (*C*), INAH-4 (*D*), and the SON (*E*).

and positron emission tomography (Stahl et al., 1986). Indeed, the link between sexual differentiation of the rat brain and that of the human brain will become clear with advancing time and technology. It is for this reason that we believe that the previously unnamed interstitial nuclei of the anterior hypothalamus (INAH) be described with this simple nomenclature and with a clear description of their anatomical locations (figures 35.1 and 35.2). Since there appears to be more than one sexually dimorphic nucleus in this region, and there is presently no indication that INAH-1 is homologous to the SDN-POA of the rat, we do not believe that it is appropriate for INAH-1 to be called the SDN-POA, regardless of its potential sexual dimorphism. Furthermore, INAH-1 extends into a region of the anterior hypothalamus that is not part of the POA (figures 35.1; 35.2, *G, J*).

### Function

Despite histological and histochemical characterization of sex differences in the MPN of the rodent, little is known about its function. Lesions of the MPOA in female rats (Powers and Valenstein, 1972) and lesions of the MPOA and particularly those including the SDN-POA (Hennessey et al., 1986) in male rats increase feminine sexual behavior. Although lesions of the POA decrease male reproductive behavior in both the rat (Arendash and Gorski, 1983) and primate (Slimp et al., 1978) specific lesions of the rat SDN-POA have not been shown to affect such behavior (Arendash and Gorski, 1983). However, SDN-POA volume positively correlates with both testosterone levels and masculine reproductive behavior in male rats (Anderson et al., 1986). Electrical stimulation of the monkey POA evokes penile erection, ejaculation, and mounting and thrusting behavior. Similarly, changes in neuronal activity in the POA of the male monkey have been related to sexual activity (Maclean and Ploog, 1962; Robinson and Mishkin, 1966; Oomura et al., 1983). Although the POA is essential for cyclic gonadotropin regulation in rodents (Gorski, 1968) it is apparently not necessary in primates; however, it probably plays a modulatory role (Plant et al., 1979; Pohl and Knobil, 1982).

### What Determines Sexual Dimorphism?

We do not know whether genomic determinants, environmental factors, and/or hormone levels influence the sex differences in INAH-2 and INAH-3. However, there is evidence that some facets of human behavior may be influenced by hormone levels during the perinatal period (Hines, 1982). In some species, structural neural sex differences are clearly affected by gonadal steroids. For example, in the rat (Gorski et al., 1978, 1980) and guinea pig (Hines et al., 1985), the steroid

environment during perinatal development appears to determine the size of the sexually dimorphic nuclei. Indeed, gonadal hormones may serve as neurotrophic substances during the development of the POAHA (Gorski, 1985). In other cases, such as the sexually dimorphic area of the gerbil and nuclei involved in song behavior in canaries, the prominence of the sexually dimorphic regions is affected by both the early hormonal environment and by adult hormone levels (Nottebohm, 1980, 1981; Commins and Yahr, 1984). In fact, steroid hormones may play a powerful role in neural plasticity in the adult brain by inducing dendritic growth and the formation of functional synapses (DeVoogd and Nottebohm, 1981; Matsumoto and Arai, 1981).

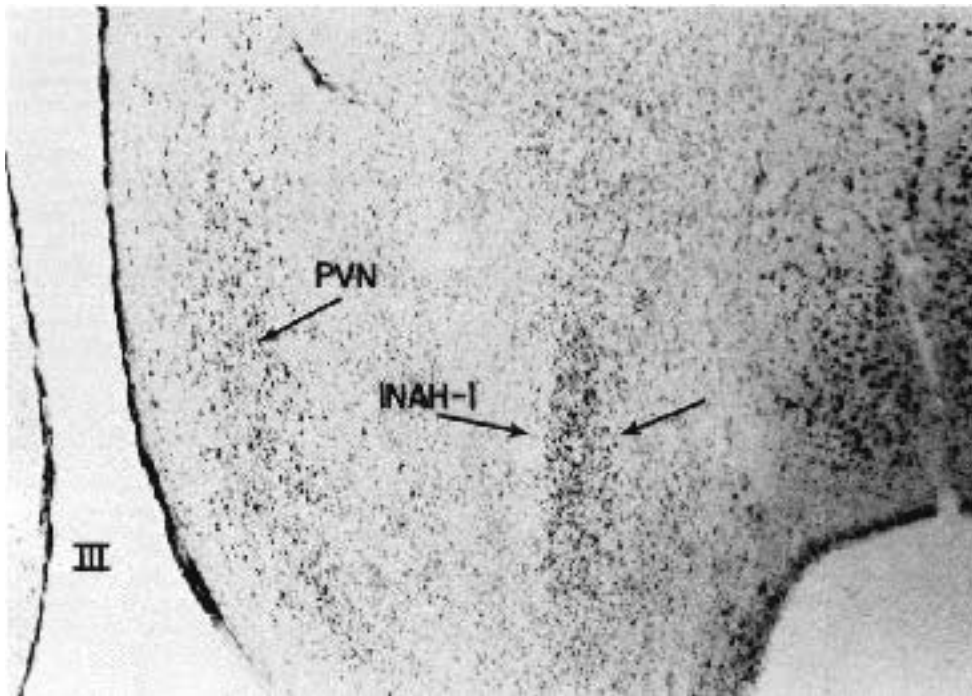
INAH-3 may resemble the SDN-POA of the rat and the guinea pig to the extent that its volume might be determined by early hormone exposure. It is consistently larger in males than in females, despite changing levels of steroid hormones throughout life. In contrast, INAH-2 may be similar to the sexually dimorphic cell groups of canaries and of gerbils in that its volume may change with fluctuations in circulating hormones. It is important to note, however, that our sample contained only 3 women of childbearing age and only 1 prepubescent pair. Clearly, further data are needed before any conclusions can be made regarding the possible influence of changing steroid titers on the volumes of INAH-2 and INAH-3.

### Asymmetry

Although complete analysis of INAH 1–4 and the SON were performed on only the right side of the brain, one investigator evaluated INAH 1–4 on both sides of the brain, and observed no pattern of asymmetry. Although no asymmetries have been observed in the SDN-POA of the rat (Hines and Gorski, 1985), sex-related cerebral asymmetries have been reported in both the rat (Diamond et al., 1983) and the human (Wada et al., 1975) which may be related to sex differences in estrogen receptor asymmetries in cortical regions (Sandhu et al., 1986).

### Is INAH-1 Sexually Dimorphic?

During the course of the present study, Swaab and Fliers (1985) reported a sexually dimorphic nucleus in the human POA that resembles INAH-1 in location, size, shape, and cell type, and in its dramatic decrease in volume with advancing age. As shown in figure 35.4, INAH-1 of a 64-year-old female brain, cut more obliquely than the other hypothalami, closely resembles—in terms of its location, relative orientation, and shape—the published photograph of what Swaab and Fliers named the “SDN-POA.” In our serial-section study of the PO-AHA, no other nuclei



**Figure 35.4**

Photomicrograph (60  $\mu\text{m}$ ) of INAH-1, from a 64-year-old female, which is in a slightly different plane of section than figures 35.1 and 35.2, appears to be the same nucleus that Swaab and Fliers (1985) named the “sexually dimorphic nucleus of the preoptic area” because of its shape, position in relation to adjacent structures, cell type, size relative to other nuclei in that region, and decrease in volume with advancing age (table 35.1).

besides INAH-1 resembled the nucleus described by Swaab and Fliers in terms of volume; INAH-1 was the largest distinct cell group besides the SON and PVN, and was several-fold larger than INAH-2–4. The fact that Swaab and Fliers reported the nucleus they studied to be 61% smaller than INAH-1 is probably due to different methods of histological preparation. INAH-1 was evaluated in gelatin-embedded 60  $\mu\text{m}$  sections, while Swaab and Fliers evaluated their tissue in paraffin-embedded 6  $\mu\text{m}$  sections. Moreover, the borders of the rat SDN-POA at 6  $\mu\text{m}$  are difficult to define and the volume appears to be approximately 60% smaller than that calculated from the 60  $\mu\text{m}$  sections we use routinely for SDN-POA volume quantification (Gorski et al., 1980).

Although we firmly believe that the nucleus studied by Swaab and Fliers is the same as INAH-1, we can only speculate as to why they determined this nucleus to be 2.5 times larger in the male brain, whereas we found that INAH-1 was only 1.2-fold larger in males than in females, prior to brain weight adjustment. Most likely, some discrepancy is due to the fact that our subjects were age-matched, with the average age of females being 2 months less than the average age of males. In contrast, in the Swaab and Fliers study, females were an average of over 10 years older than the males. Since INAH-1 decreases with age, this age

difference may have contributed to the significant differences between their male and female groups. The 2 methods of volumetric quantification were also markedly different. While we had 3 independent investigators quantify serial 60- $\mu\text{m}$ -thick sections with an average of 29.5 sections per individual INAH-1, Swaab and Fliers quantified the areas of every 25th 6- $\mu\text{m}$ -thick section, except at the rostral and caudal ends of the nucleus, where every 5th section was measured, for an average of 11 sections actually evaluated. In addition, different population samples may have contributed to different results. In studies of the rat, for example, different results are sometimes obtained in different strains of animals and with different rearing conditions. Although it is not possible to match all variables in human research as well as it is in animal research, we have included information as to race as it appeared on the autopsy report (table 35.1) since this factor can influence the results.

Alternatively, it is possible that, although the volume of INAH-1 is not sexually dimorphic in 60  $\mu\text{m}$  sections, there is a sexually dimorphic subcomponent of INAH-1 that, because of greater cell density, may have been the only component of INAH-1 visible in the 6  $\mu\text{m}$  sections examined in the Swaab and Fliers study. However, in the rat, although there are sexually dimorphic subcomponents to the SDN-POA, the

volume of this nucleus is approximately the same fold larger in males than in females, irrespective of whether 60 or 6  $\mu\text{m}$  sections are analyzed. We plan to examine cell density in a further study of the INAH.

### Conclusion

Because the human being cannot be manipulated experimentally as can laboratory animals, it is difficult to extrapolate from animals to humans regarding structural, behavioral, or physiological sex differences. It is interesting to speculate that factors such as prenatal stress that both feminize and demasculinize sexual behavior (Ward, 1984) and decrease the volume of the SDN-POA in male rats (Anderson et al., 1986) may, similarly, contribute in human males to homosexuality (Dörner, 1976) and to a decrease in the volume of the sexually dimorphic INAH; moreover, the INAH are located in a region of the brain influencing sex differences in gonadotropin secretion which may be altered in some homosexual men (Dörner, 1976; Gladue et al., 1984). Furthermore, gonadal hormones may be important neurotrophic factors in both the developing and adult human and, in fact, have been shown to improve symptoms of Alzheimer's disease in some postmenopausal women (Fillit et al., 1986). Thus, morphological analysis of the brains from humans with different sexual orientations and identities, during different stages of development, and from individuals exposed perinatally to atypical steroid hormones or therapeutically to altered adult hormone levels may lead to further deductions concerning the possible influence of sex hormones on the structure and function of the human brain.

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The results of a number of studies in which various experimental approaches were utilized, have strongly implicated the medial preoptic area (MPOA) in the regulation of sexual behavior in the male rat. Large, bilateral lesions within this region greatly impair or eliminate copulatory behavior [2, 9, 10, 15, 19, 23, 26], while electrical stimulation of the MPOA can substantially enhance this behavior [20, 21, 27]. Furthermore, the MPOA in male rats has very high uptake and retention of radioactively labelled testosterone [24]. In this regard, implants of crystalline testosterone into the MPOA can partially restore sexual behavior in castrated male rats [1, 7].

Since large MPOA lesions drastically reduced male copulatory behavior, Heimer and Larsson [15] attempted to define further a critical area within this region by making smaller MPOA lesions. They reported a temporary decline in various aspects of sexual behavior after some lesions, but not related to the obliteration of any specific part of the MPOA. In a later study, Christensen et al. [2] found that male rats subjected to bilateral lesions restricted basically to the dorsal MPOA (but still destroying at least 50% of the MPOA) showed little or no post-lesion copulatory behavior. This last finding, when coupled with the observation of a sexual dimorphism in synaptic connections within the dorsal preoptic area of the rat [22], may suggest a relatively more important role for the dorsal MPOA than other preoptic regions in male sexual behavior.

Recently, this laboratory identified another morphological sex difference within the MPOA—observable even without magnification [11, 12]. This intensely staining region, located just below the dorsal MPOA and now called the Sexually Dimorphic Nucleus of the Preoptic Area (SDN-POA) [13] has a volume five times larger in the adult male compared with that in the female. Furthermore, this sexual dimorphism in volume is apparently independent of gonadal hormones in the adult, but critically dependent upon the perinatal hormonal environment [5, 12]. The role, if any, of the SDN-POA in reproductive processes has yet to be determined.

The purpose of the present study was to determine the relative importance of the SDN-POA and other specific regions within the MPOA for the expression of male sexual behavior. Therefore, discrete lesions were placed within the SDN-POA, dorsal MPOA, or ventral MPOA of adult male rats and their effects on male copulatory behavior evaluated.

## Method

### Experimental Animals

Adult male Sprague-Dawley rats (Simonsen Laboratories) weighing 300–380 g were maintained in a reverse light room (lights off from 12:00 noon to 10:00 P.M.) with access to Purina rat chow and water ad lib. Prior to behavioral testing animals were given sexual experience with receptive females in 3 two-hour sessions at 3 day intervals. All rats were screened for masculine sexual behavior on three ensuing 20 minute tests at 3 day intervals, and only those animals which ejaculated at least twice during two of these pre-experimental tests were included in the study. Approximately 60% of the animals screened met this criterion, and there were no differences in pre-surgery ejaculatory behavior between the various groups formed for the actual study.

### Surgery

Following the screening tests, small electrolytic lesions (anodal DC, 1 mA for 3–4 sec) were placed bilaterally within the MPOA in animals previously anesthetized with sodium pentobarbital (Diabital, 35 mg/kg bw IP). Lesions were produced using an electrode constructed from 0.10 in. platinum wire inserted through a segment of 28 g stainless steel tubing, with the entire electrode apparatus being insulated with epoxylite except for 0.3 mm at the tip of the exposed platinum wire. With the stereotaxic atlas of König and Klippel [18] as a guide, lesioning coordinates were: 7.8–8.3 mm anterior to the interaural line, 0.5 mm lateral, and 7.7–8.2 mm below the dura. Sham procedures were identical except that no current was passed. Immediately after lesioning and prior to recovery from pentobarbital anesthesia, all animals were bilaterally castrated

utilizing a transscrotal approach. Each animal concurrently received a 15 mm testosterone-filled Silastic capsule implanted subcutaneously. These capsules have previously been shown to maintain copulatory behavior at normal levels for well over one month after implantation into castrated rats [6]. The gonadectomy and capsule implantation procedures were done to circumvent any possible indirect effects of MPOA lesioning on sex behavior through impaired testosterone secretion.

### Copulatory Tests

Tests of copulatory activity were conducted every third day beginning 5 days after lesioning and continuing through post-lesion day 17, although testing of some animals was continued at the same interval for one month after lesioning. Testing generally started at 1:00 P.M. and began by placing the male alone into a Plexiglas area (46 cm × 46 cm) for a 10 minute adaptation period. A receptive female (previously spayed and primed with estradiol benzoate plus progesterone) was then introduced to begin observation and timing of the behavioral test, with each test lasting 20 minutes. Receptive females were changed midway through this period. The following measures of male copulatory activity were made: (1) Number of mounts: The total number of mounts observed during the entire 20 minute test; (2) Number of intromissions: The total number of intromissions occurring for the entire test; (3) Number of ejaculations: The number of ejaculations exhibited during the entire test; (4) Intromissions to ejaculations: The number of intromissions seen between the start of the test and the first ejaculation; (5) Time to first event: The time interval between introduction of the receptive female and the occurrence of the first mount or intromission; (6) Time to first intromission: The time between introduction of the receptive female and the first intromission; (7) Time to first ejaculation: The time interval between introduction of the receptive female and the first ejaculation; (8) Post-ejaculatory interval: Time from first ejaculation to the first ensuing intromission.

### Histology and Statistics

Upon completion of all behavioral tests, animals were injected with pentobarbital and then perfused with 10% formalin solution. Brains were removed and stored in formalin solution for at least three days, after which they were sectioned serially at 50  $\mu$ m, mounted, and stained with thionin. Histological examination served to classify animals into one of four MPOA-lesioned groups or one sham-lesioned group. The data from experimental animals was then grouped accordingly and analyzed by analysis of variance and Duncan's Multiple Range Test or the unpaired *t*-test (two tailed).

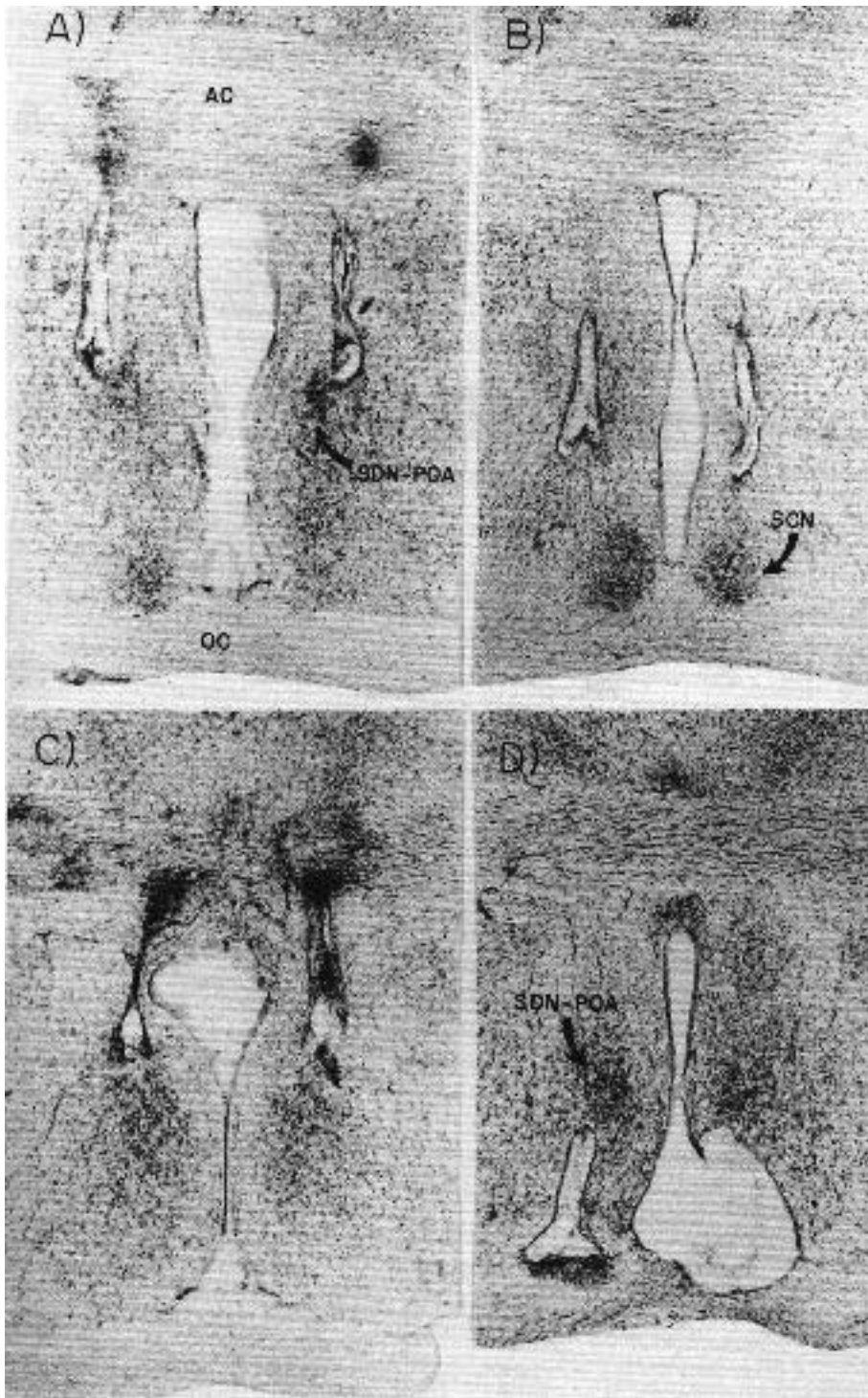
## Results

### Histological Examination

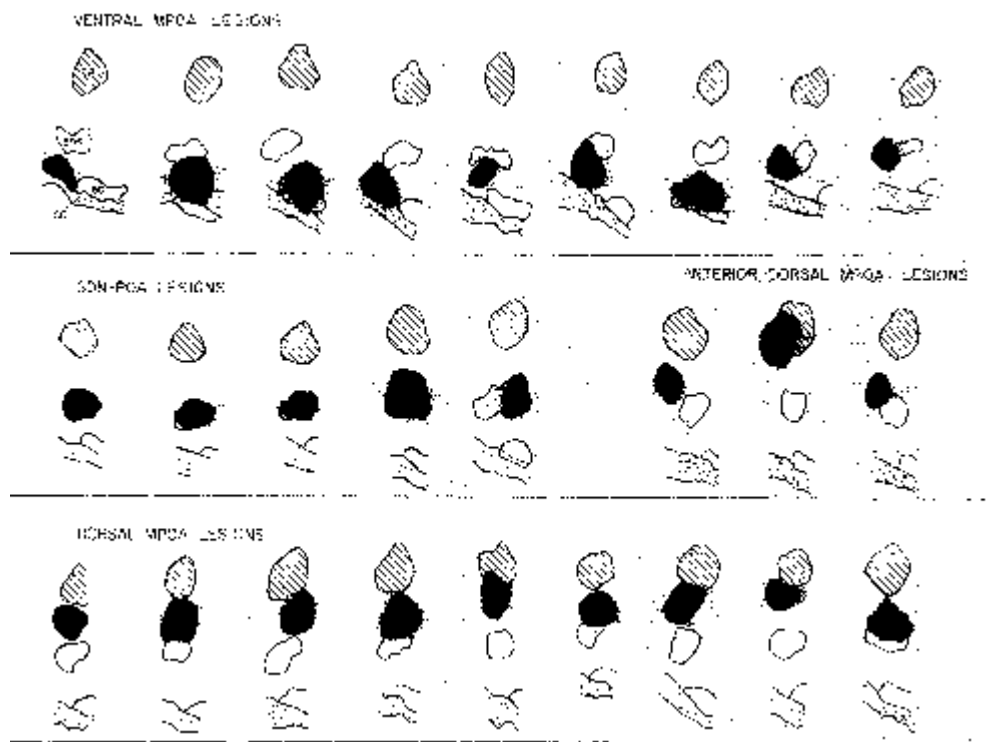
A total of four separate and discrete lesion groups were derived from histological examination of the brains: Ventral MPOA (V-MPOA), SDN-POA, dorsal MPOA (D-MPOA), and antero-dorsal MPOA (Ant. D-MPOA). Representative lesions are shown in figure 36.1. V-MPOA lesions were located in the ventral third of the MPOA, generally just anterior to the suprachiasmatic nucleus, although several lesions impinged to varying degrees on this nucleus. SDN-POA lesions involved complete or almost total destruction of this structure, located midway between the anterior commissure and the optic chiasm. Very little tissue surrounding the SDN-POA was included in these lesions. D-MPOA lesions were localized to the dorsal third or half of the MPOA, immediately beneath the anterior commissure and dorsal to the SDN-POA. Animals in the Ant. D-MPOA group had more anteriorly placed dorsal lesions, which did not destroy the area immediately beneath the anterior commissure. Sagittal reconstructions of MPOA lesions for all animals included in the above four groups are depicted in figure 36.2. As can be seen from this figure, most lesions were quite similar in size and all were small.

### Copulatory Behavior

Initially, an analysis of variance (ANOVA) for each group across the 5 post-lesion tests revealed no effect of time on number of mounts, intromissions, and ejaculations. Therefore, a "mean" number for each of these three parameters could be obtained in the case of each animal by averaging the respective values recorded for all 5 tests. Graphs of the resulting "mean" number of mounts, intromissions and ejaculations calculated for each group are shown in figure 36.3. A between-groups ANOVA revealed significant ( $p < 0.001$ ) decreases in the mean number of mounts and intromissions exhibited by D-MPOA lesioned animals compared with sham-lesioned controls. Neither of these parameters was affected by lesions placed in the Ant. D-MPOA, SDN-POA, or V-MPOA. Although an overall between-groups ANOVA failed to detect any significant differences among the experimental groups with regard to mean number of ejaculations (perhaps due to the low incidence of this behavior), a highly significant ( $p < 0.001$ ) decrease in ejaculations was observed for D-MPOA lesioned animals when their performance was compared directly to that of the sham-lesioned control group. A similar, though less dramatic ( $p < 0.02$ ) decrease in the mean number of ejaculations was also seen for the V-MPOA lesioned group, compared to that of sham-lesioned controls; animals with SDN-POA or Ant. D-MPOA lesions

**Figure 36.1**

Representative photomicrographs of coronal brain sections through the medial preoptic area of four lesioned rats showing typical dorsal MPOA lesions (*A*), SDN-POA lesions (*B*), antero-dorsal MPOA lesions (*C*), and ventral MPOA lesions (*D*). Abbreviations: AC, anterior commissure; SCN, suprachiasmatic nucleus; SDN-POA, sexually dimorphic nucleus of the medial preoptic area; OC, optic chiasm.



**Figure 36.2**

Sagittal reconstruction of medial preoptic area lesions for all animals in all four lesion groups. Abbreviations: CA, anterior commissure; CO, optic chiasm; SC, suprachiasmatic nucleus; SDN-MPOA, sexually dimorphic nucleus of the medial preoptic area.

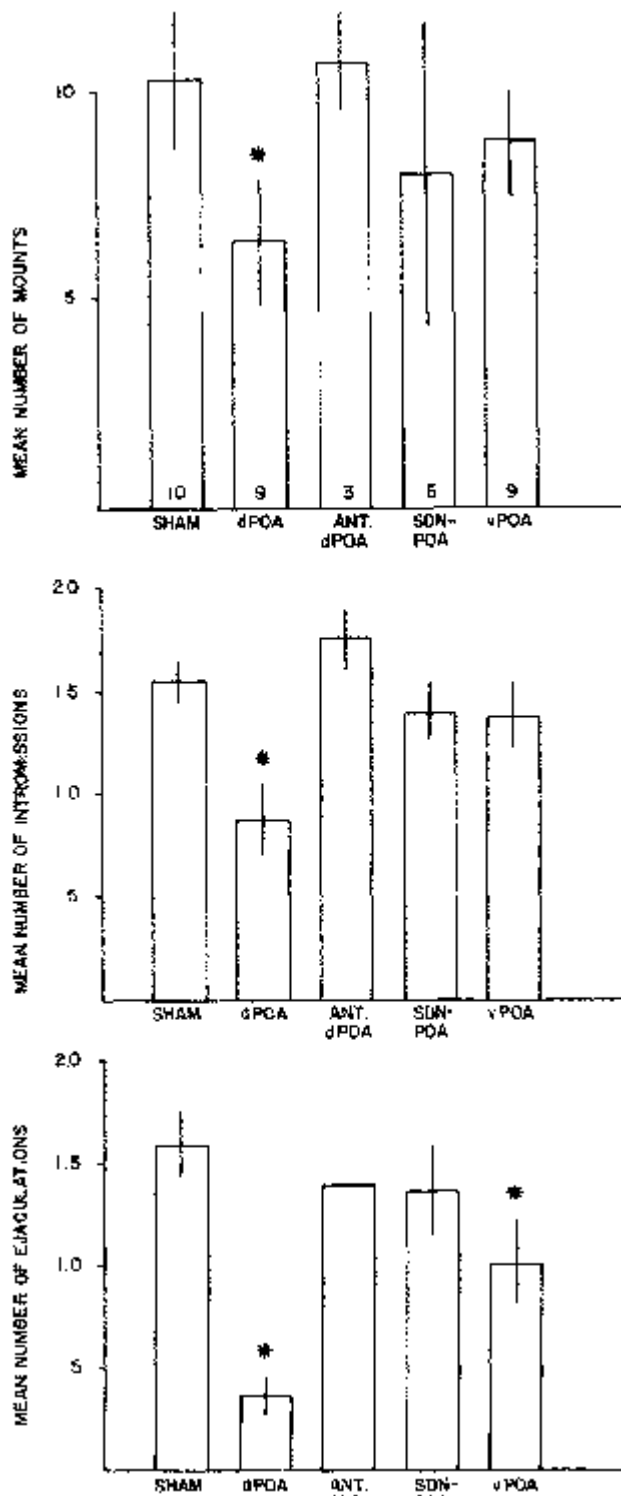
showed ejaculatory frequencies similar to that of sham-lesioned rats.

The apparent deleterious effects of D-MPOA lesions on ejaculatory responses are further emphasized by figure 36.4, which shows the percentage of animals in each experimental group that ejaculated in each of the 5 post-lesion tests. On the first post-lesion tests, only 11% (1/9) of D-MPOA lesioned animals ejaculated. In the second test, the percentage of D-MPOA animals ejaculating increased somewhat (to 33%) and remained at about this level for the remaining three tests. In an attempt to observe any late recovery of copulatory activity, three D-MPOA lesioned animals were tested an additional 4 times (through post-lesion day 29). Behavioral scores for these animals remained at low levels, indicating that no recovery from lesioning occurred. Furthermore, two animals in the D-MPOA group never ejaculated during any post-lesion test; one of these became completely sexually inactive. Although V-MPOA lesioned rats when compared directly with the sham-operated controls showed a significant decrease overall in mean number of ejaculations, Figure 36.4 indicates that this effect was not reflected in a substantial decrease in the percentage of V-MPOA animals ejaculating.

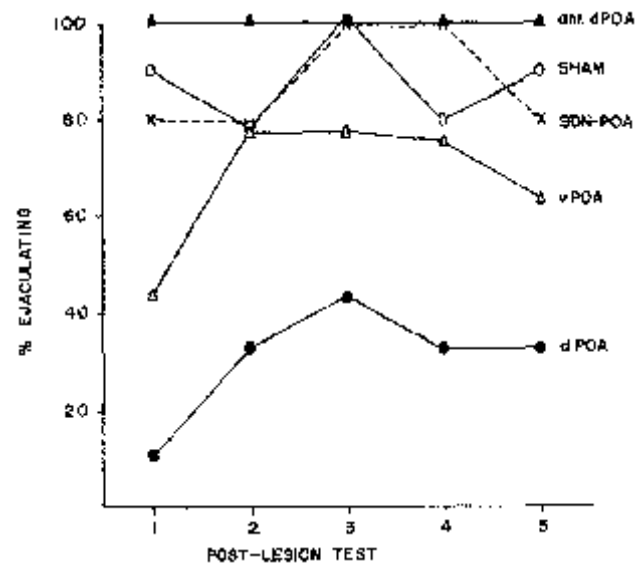
For the 5 experimental groups of this study, an overall analysis of variance across the 5 post-lesion tests re-

vealed no significant effect of time on the remaining five parameters of copulatory behavior. Therefore, for each animal, a "mean" number for each of these five parameters was obtained by averaging the respective values over the 5 test sequence. Table 36.1 shows the resulting average time (in seconds) to the first event, intromission, and ejaculation for each group, as well as their respective mean number of intromissions in the first ejaculation and mean post-ejaculatory interval. Analysis of variance for each of these five parameters showed no between-group differences, thus indicating that no lesion group was significantly different from the sham-lesioned control group for any of the 5 parameters.

Data collected in D-MPOA animals for the above 5 parameters may be deceiving, however, since such "timed" parameters were recorded only if they had been observed for an animal. In the first two post-lesion tests, the percent of D-MPOA animals showing a mount or intromission was only 44 and 56%, respectively, which was considerably below the 78–100% of animals in the other 4 experimental groups showing these behaviors. Likewise, only about 33% of D-MPOA rats ejaculated on any single test. Nevertheless, for those D-MPOA animals that did show mounts, intromissions, and/or ejaculations, the latency to these events was not consistently increased above sham group latencies throughout the 5 test sequence.

**Figure 36.3**

Mean number of mounts, intrusions, and ejaculations displayed over a 5 test sequence by male rats previously lesioned within the dorsal MPOA (dPOA), antero-dorsal MPOA (ANT.dPOA), sexually dimorphic nucleus of the MPOA (SDN-POA), or ventral MPOA (vPOA). \*Indicates significantly different ( $p < 0.02$ ) from sham group.

**Figure 36.4**

Percentage of animals in each experimental group that ejaculated during each of 5 post-lesion tests. One way ANOVA after arc sine transformation of the mean percentage of rats exhibiting ejaculatory behavior across groups revealed a highly significant ( $p < 0.001$ ) group effect. Abbreviations: same as figure 36.3.

### Discussion

The prevailing concept of the role of the medial pre-optic area (MPOA) in the regulation of male copulatory behavior assumes that this structure is critically important for the initiation and expression of male sexual activity since large MPOA lesions greatly impair or eliminate copulation [2, 9, 10, 15, 19, 23, 26]. The current study extends and refines this concept by indicating first, that small discrete lesions encompassing the SDN-POA have no effect on the maintenance of copulatory behavior in the rat. Secondly, lesions of similar size placed within the dorsal MPOA can substantially disrupt various aspects of masculine sexual behavior. Specifically, the decremental effects induced by dorsal MPOA lesions appear to involve ejaculatory mechanisms (i.e., those which mediate the number of mounts, intrusions, and ejaculations displayed) rather than initiation (arousal) mechanisms (i.e., those which mediate time to the initial mount or intrusion).

The inability of lesions encompassing the SDN-POA to affect copulatory activity is of particular interest. The volume of this nucleus—being about 5 times larger in the adult male than female—is highly dependent on the perinatal hormonal environment. Neonatally castrated male rats have a significantly reduced SDN-POA volume when they reach adulthood, whereas neonatal females injected with testosterone propionate have a significantly larger SDN-POA as

**Table 36.1**

Effects of small lesions within the MPOA on various aspects of copulatory behavior in male rats averaged over 5 post-lesion tests

Lesion Group	T-EV	T-I	T-E	I-E	PEI
Sham	120 $\pm$ 33*	149 $\pm$ 38	563 $\pm$ 63	11.5 $\pm$ 0.8	378 $\pm$ 24
D-MPOA	168 $\pm$ 61	246 $\pm$ 70	743 $\pm$ 92	12.2 $\pm$ 1.2	407 $\pm$ 17
Ant. D-MPOA	28 $\pm$ 12	54 $\pm$ 6	525 $\pm$ 24	13.1 $\pm$ 0.9	421 $\pm$ 7
SDN-POA	62 $\pm$ 26	103 $\pm$ 44	591 $\pm$ 52	10.4 $\pm$ 0.8	382 $\pm$ 38
V-MPOA	140 $\pm$ 47	195 $\pm$ 65	726 $\pm$ 68	13.1 $\pm$ 1.4	399 $\pm$ 20

\* Values are mean  $\pm$  SE. All parameters, except I-E, are in seconds.

Abbreviations: T-EV: Time to first event; T-I: Time to first intromission; T-E: Time to first ejaculation; I-E: Number of intromissions to first ejaculation; PEI: Post ejaculatory interval.

adults [12]. In fact, treatment both pre- and postnatally with this steroid completely masculinizes SDN-POA volume in the female [5]. Consistent with the view that this nucleus undergoes sexual differentiation are the findings that (1) there may be sex differences during gestation in neuronal production for neurons destined for the SDN-POA [17]; and (2) cells which eventually comprise the fetal SDN-POA continue dividing for several days after the rest of the preoptic area has apparently become post-mitotic [17]. Yet, the results of the present study indicate that the integrity of the SDN-POA is not critical for a full expression of male sexual behavior. If the SDN-POA is involved in the regulation of masculine sexual behavior, its role must be subtle and of less importance than that of the D-MPOA. In addition, the present experiment was designed to investigate the "maintenance" of male copulatory behavior. Perhaps significant effects of SDN-POA lesions would have been observed had the androgen dependent "restoration" of this behavior been evaluated following its disappearance after castration. The neurons of the SDN-POA obviously may play an integral role in other hormonal regulatory (e.g., prolactin release [4]) or homeostatic mechanisms.

In contrast to the inability of small discrete SDN-POA lesions to affect male sexual behavior, lesions placed within the D-MPOA resulted in marked impairments of this behavior. Similar small lesions placed in the Ant. D-MPOA and V-MPOA (in addition to the SDN-POA) were not effective in consistently reducing sexual behavior, thus indicating that the D-MPOA may be differentially more important than these other MPOA regions for copulatory activity. In a study reported by Christensen et al. [2], initial evidence for the importance of the dorsal MPOA in male sexual behavior was obtained. These authors found that lesions restricted to the dorsal half of the MPOA (but still involving at least 50% of the entire MPOA) resulted in very little or no male sexual behavior. Although the copulatory deficits produced by D-MPOA lesions in the present study are not quite as dramatic as those reported in this earlier study, lesion size appears critical

in determining the severity of impairment; clearly, the dorsal MPOA lesions of the present study were much smaller and more discrete.

In this report, dorsal MPOA lesions impaired the "initiation" (sexual arousal) mechanism(s) temporarily, while causing apparently long-term deficiencies in the "ejaculatory" mechanism(s). With reference to the former effect, the number of D-MPOA lesioned animals that initiated copulatory behavior (i.e., showed at least a mount or intromission) on the first two post-lesion tests (Days 5 and 8) was a low 44 and 56%, respectively. On the next day (Day 11), however, 89% of animals in this group initiated copulatory behavior. Furthermore, latencies to the first copulatory event (mount or intromission) for D-MPOA lesioned rats did not differ from shams overall, throughout the 5 test sequence. Therefore, the early impairment of initiation mechanisms in the D-MPOA group was temporary in nature. Such was not the case for the ejaculatory mechanism(s) of D-MPOA lesioned animals, however, since they consistently had lower numbers of mounts, intromissions, and especially ejaculations throughout the entire testing sequence (which was up to one month for some animals). No recovery from the decremental effects of D-MPOA lesions on these three parameters was observed.

At present, one can only conjecture as to the nature of the neuronal pathways important for copulation that are disrupted by D-MPOA lesions. The stria terminalis has been shown to project both a postcommissural and a precommissural component to the medial preoptic-anterior hypothalamic area [16]. The post-commissural component terminates massively in the rostral part of the anterior hypothalamus, while the precommissural component simply transverses the MPOA en route to termination points around the ventromedial hypothalamus nucleus. Interestingly, lesions of the corticomedial amygdala [14], the bed nucleus of the stria terminalis [8], or the stria terminalis itself [9], have all been shown to increase the time required to reach ejaculation; lesions of the first two sites also increased the number of intromissions required for an

ejaculatory response. No such alteration in the ejaculatory mechanism was observed for those D-MPOA lesioned animals of the present study that did ejaculate (i.e., time to ejaculation and number of intromissions to ejaculation were both comparable to those of sham controls). Therefore, the qualitatively different behavioral deficits in the ejaculatory mechanism of D-MPOA lesioned rats may not involve components of the stria terminalis projection system. Indeed, the specific and consistent location of D-MPOA lesions just ventral to the anterior commissure would indicate little, if any, lesion-induced destruction to the postcommissural stria component [16]. Still, if the individual tests had been of longer duration, more D-MPOA lesioned animals possibly would have ejaculated and with a copulatory pattern similar to that of rats with stria terminalis or bed nucleus lesions. Although some destruction to the precommissural stria component undoubtedly occurred in D-MPOA, Ant. D-MPOA, and SDN-POA lesioned animals, this projection may not be modulatory to copulation since coronal MPOA knife cuts placed just rostral to the anterior commissure [25] or large anterior MPOA lesions [26] do not affect male sexual behavior in rats.

Searching for a locus within the medial preoptic-anterior hypothalamic region that might control male copulatory behavior, Heimer and Larsson [15] produced relatively small lesions within this area. They reported that none of these lesions eliminated sexual behavior and those that did impair behavioral performance were not restricted in location to any specific part of the medial preoptic-anterior hypothalamic area. These authors therefore conclude that "the neurons ultimately responsible for the mating behavior are dispersed over a relatively wide area including the whole medial preoptic-anterior hypothalamic region." The results reported in the present study do not conflict with this view, nor do they totally conform with it. Certainly, none of the four MPOA lesion groups of this study had a complete abolition of copulatory activity. However, D-MPOA lesioned animals did have selective and consistent impairments of their ejaculatory mechanism, thus indicating a possible greater importance of this MPOA region in male sexual behavior than other MPOA areas. Indeed, in a now classic study, Raisman and Field [22] demonstrated a sexual dimorphism in the non-stria synaptic connectivity within the dorsal MPOA, immediately ventral to the anterior commissure. They observed that females had a much larger region of the dorsal MPOA containing a high density of spine synapses than did males. It is interesting to note that, in the male, the small patch of neuropil containing this high density of spine synapses (see [22]) was an area common to all D-MPOA lesions in the present study. In further support of the impor-

tance of the dorsal MPOA in copulation, neonatal implants of testosterone or estradiol into the dorsal MPOA of female rats have been shown to increase significantly the amount of masculine sexual behavior displayed by these animals as adults [3].

In conclusion, the present data support the concept of functional specialization on an anatomical basis within the MPOA, at least in terms of the regulation of male sexual behavior in the rat. Additional studies, utilizing a variety of neuroendocrine and anatomical techniques, appear warranted at this time to further explore discrete effects within the MPOA on various neuroendocrine regulatory mechanisms.

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## Introduction

Electrical stimulation of specific loci in the brain will produce a variety of autonomic responses involving male sexual organs. Effects observed in rats and monkeys include: penile erection (20, 35), seminal discharge (19, 38) and ejaculation (15, 25, 33). The relevance of the observed responses to sexual behavior was not investigated in those studies. A number of experiments have successfully demonstrated activation or facilitation of copulatory behavior when the males were stimulated in the presence of receptive females. However, interpretation of these findings presents certain problems. In most studies, sexual responses were obtained from only a small percentage of subjects (24, 39, 40). Many of the reported stimulus-bound effects represented only components of the mating behavior of the subject animal. For example, sexual display and mounting behavior can be elicited by hypothalamic stimulation in male opossums; however, penile erection or intromission did not accompany the mounting response (32). In some studies, although stimulation did not induce mounting behavior at a fixed short latency, male sexual behavior was enhanced when stimulation was applied in trains of 30 sec or longer during spontaneous copulation (21, 22).

Other investigators have reported success in eliciting sexual responses that were stimulation-bound (4, 24, 40). These effects were produced in male rats by delivering stimulation trains of 1–3 min durations, presented at intervals of equal duration. A series of mounts, with or without intromission, occurred during stimulation but spontaneous sexual behavior was suppressed between stimulus trains (6, 24). As with facilitation effects, the relationship between stimulus onset and mount latency was variable. The number of mounts and the intermount intervals during the stimulus train differed from trial to trial.

We have reported previously that stimulus-bound mounting behavior can be induced by stimulation of the rostral putamen and the hypothalamus in male rhesus monkeys (26, 27, 29, 30). These responses were characterized as sexual in nature since the stimulated

males preferentially mounted females and since intromission, thrusting and, in some cases, ejaculation occurred during the stimulus-induced mount. The experiments described in the present report provide additional evidence from a larger subject population that indicates the importance of two regions for stimulation-produced mounting behavior: the preoptic area and the dorsomedial nucleus of the hypothalamus (DMH). In a small number of subjects, from which a large number of observations was obtained, quantitative measures of specific aspects of sexual performance were made to distinguish significant differences between behavioral responses to stimulation of these two anatomical areas. Finally, we describe the behavioral specificity of the response to stimulation as well as the relationship between elicited penile erection and mounting behavior.

## Methods

Adult male rhesus monkeys were tested for their ability to exhibit mature copulatory behavior as described by Goy and Goldfoot (11) when paired with estrogenprimed females. After an adaptation period of at least 5 h long observation sessions, 11 males ranging in weight from 7 to 10 kg were chosen for the study. Adult rhesus females were bilaterally ovariectomized and prepared for mating tests by treatment with subcutaneous administration of 17- $\beta$ -estradiol-3-benzoate (Sigma Chemical Co.), either by injection of 30  $\mu$ g of the steroid suspended in medium chain triglycerides oil (Mead-Johnson), three times per week, or by the implantation of silastic capsules containing the crystalline form of the hormone.

## Implantation Procedures

An electrode guide platform was stereotactically placed on the skull of the anesthetized monkey so that the stimulating electrodes would be oriented toward the preoptic area and the hypothalamus. The platform was constructed of Delrin, which was selected for its strength, machinability and biological inertness. A grid pattern of holes was drilled at 1 mm intervals.

Although electrodes were never placed that closely together, this spacing allowed correction of errors in stereotaxic alignment of the guide holes with respect to structures of interest. Since adult rhesus monkeys exhibit significant anatomical variability, several procedures were developed to ensure the proper placement of electrodes in chosen hypothalamic and preoptic structures. Lateral and anterior/posterior radiographs were made of the cranium of the anesthetized animal. Stainless steel pins, placed in selected holes in the platform, produced radio-dense images on the X-ray film that were used to project electrode paths through the brain toward target sites. Anatomical features of the base of the skull visible in radiographs taken from the lateral view (e.g., sella turcica and the optic foramen) were used to locate structures in and around the preoptic and medial basal hypothalamus. When the appropriate guide holes were selected, the bone and dura were penetrated with a sterilized drill. The holes were sealed with sterile stainless steel pins to maintain their patency until the electrodes were inserted.

One to two weeks following surgery, animals were placed in primate restraint chairs and electrodes were implanted. As the electrodes were lowered into the brain, impedance measures were made in order to detect major anatomical landmarks (33). This method was particularly useful for locating the preoptic area which is bound by high impedance, myelinated fibers in the anterior commissure dorsally and the optic chiasm ventrally. Beginning ventral to the corpus callosum, test trains of stimulation were delivered at each millimeter during the vertical traverse of the electrode. Stimulation consisted of 10–30 sec duration trains of monophasic, cathodal square waves of either 0.1 or 1.0 msec pulse duration, at 50 Hz pulse repetition rate, ranging from 0.1 to 0.8 mA peak pulse current. Current and the tissue/electrode interface impedance were simultaneously monitored through high impedance differential amplifiers in a Wheatstone Bridge.

Concentric bipolar electrodes were constructed from stainless steel tubing and wire. The surround was constructed with 28-gauge hypodermic tubing insulated with epoxylite to an overall diameter of 0.4 mm. The core wire was an insulated 100  $\mu$ m wire inserted through the lumen of the tubing, extending 1.0 mm beyond its tip. The exposed stimulating surfaces of each pole measured 0.5 mm in length. In order to minimize polarization of the electrode tips, the exposed stainless steel surfaces were gold-flashed and then platinized. Each pole of these electrodes was stimulated independently by employing a “monopolar” electrode configuration. One of the 4 stainless steel bolts that secured the platform to the skull acted as the anode. Thus, for each electrode track, two sites, separated by approximately 1 mm in the vertical plane, could be tested separately.

### Remotely Controlled Stimulation

Each implanted male monkey was adapted to wearing a head-mounted, radio-controlled stimulator. Electrical connection to each of the electrodes was made through mating plugs and sockets incorporated in the platform. The animal-worn stimulator produced pulse trains composed of the same stimulus parameters that were used while implanting the electrodes in the restrained animal. The instrument is designed to enable the experimenter to select one of 4 electrodes for stimulation and to control independently the pulse repetition rate, the pulse duration, the pulse current and the length of the stimulus train. A complete description of this system has been published elsewhere (31). The monkeys adapted readily to the stimulator unit. No attempts were made by the implanted animal or by other members of the group to manipulate or remove the instrument. Proper functioning of the stimulator was ensured by two means. (1) Calibration of the stimulator prior to and following test sessions verified the accuracy of the selected stimulus parameters. (2) The head-mounted unit contains a radio transmitter that broadcasts a signal simultaneously with the delivery of each pulse of the stimulus train. This pulse is transmitted to a ground receiver providing information on the performance of the unit.

### Behavioral Observations

**(A) Restrained Subjects** Behavioral observations were made of the effects of stimulation at the time of implantation of each electrode. Since the animals were restrained in a primate chair for these tests, emphasis was placed on the location of sites that, when stimulated, produced a behavioral response that might have some relevance to the social behaviors that would be observed while the animal was stimulated in a social test setting. For the purposes of this study, particular attention was paid to the motor and autonomic responses of the genitals and pelvic region, including: abduction of the thighs, penile tumescence, rhythmic pelvic movements or scratching directed toward the genitals. Other motor responses that were noted included changes in facial expressions and repeated movements of the head, limbs and eyes. Both edible and inedible objects were offered to the animals during stimulation. Notes were made on the type and timing of vocalizations occurring both during and immediately following each train of stimulation. If seminal emission or ejaculation occurred with stimulation, attempts to re-stimulate that site were resumed after a lapse of at least 20–30 min. When a response of interest was elicited, the electrode was permanently fixed in place by cementing the shaft to the platform.

**(B) Unrestrained Subjects** Each male was assigned to a social group. Typically, these groups were composed of the implanted male, an estrogen-treated, ovariectomized female and another male that was usually subordinate to the implanted animal. Observations were made of the stimulated animal in this social group or only with the female present. For this purpose the animals were placed in a fiberglass cage,  $2.6 \times 1.7 \times 1.4$  m, with a glass front that was located in a radio-frequency shielded room. Behavioral observations of the group or the test pair were made through one-way screens and a closed circuit video tape system. The animals were observed for hour-long sessions during which no attempts were made to apply stimulation. Sufficient time was allowed for the animals to establish stable social relationships. The social status of each animal was determined by frequency measures of dyadic interactions.

Spontaneously occurring sexual behavior was measured during the familiarization period and throughout the experiment. Data obtained from non-stimulation sessions provided a baseline measurement for comparison with stimulation-elicited behavior. Sexual performance was quantified by the following measures: number of mounts per session, mount duration, number of thrusts per mount, number of thrusts per mount duration (thrusts per second or thrusting rate), inter-mount interval, duration of ejaculatory sequences, inter-sequence intervals (refractory periods), latency to mount and total number of ejaculations per session. An ejaculatory sequence was defined as a series of mounts beginning with the first mount accompanied by intromission and thrusting and ending with the mount during which ejaculation occurred. A measure of the social status of each animal was in part provided by measures of agonistic behaviors, including threats, chases, punishing attacks and submissive responses to aggression. In addition, proximity of animals to each other and grooming behavior were noted.

**(C) Elicited Mounting Behavior** Stimulation was applied by activating the remotely controlled stimulator aperiodically throughout an hour-long test session. Because of possible confounding of stimulus-induced behavior with spontaneously occurring sexual activity, a restricted test regimen was employed and eventually applied for the investigation of all test sites discussed in this report. For each stimulation test session, the implanted male was placed in the cage with an estrogen-treated female and allowed 5 min to exhibit spontaneous sexual behavior. If none occurred, the first train of stimulation was applied at the sixth minute, usually for a duration of 15 sec at a current level that did not exceed 0.2 mA peak current. If the pair exhibited spontaneous copulation, stimulation was delayed

until 1 min following the occurrence of ejaculation. The inter-stimulus train interval was 1 min or longer. During initial investigation of each electrode site, stimulus parameters were varied up to a maximum of 15 sec duration train of pulses at 50 Hz, 1 msec pulse duration, 1 mA peak pulse current.

Stimulus-induced mounting behavior was defined by both the temporal relationship of the response to the stimulus and the reliability of the response upon repeated stimulations. According to these criteria, a mount was classified as stimulus-related if it occurred during the stimulus train and if it recurred with subsequent stimulations applied during the session. The timing of stimulation was not systematically manipulated; thus, for the elicited behavior no significance was attached to measures of inter-mount interval or duration of elicited ejaculatory sequences. An equal number of tests was not applied to all electrode sites. Preferential treatment was given to those sites that produced reliable mounting responses at the lowest stimulus current levels. In a selected number of subjects, a protracted test series was employed in order to obtain a statistical measure of the stimulus effects on measures of sexual performance. Some of these animals were involved in weekly tests that extended for a period in excess of one year. These tests continued as long as there was no increase in the current required to obtain reliable responses.

Statistical analyses for behavioral measures included two-tailed *t*-tests for significant differences between group means for stimulation and non-stimulation conditions and Chi-square tests of goodness-of-fit for comparisons among response types.

### Histology

At the end of the experiments each male was sacrificed with a lethal dose of sodium pentobarbital. The animals were perfused through a catheter placed in the ascending aorta, first with normal saline followed by a formalin/saline solution. Following fixation, the brain was allowed to harden in situ. The tissue was blocked in stereotaxic planes, removed from the cranium and submerged in a 20% sucrose-formalin solution for one week. Frozen sections, 40  $\mu$ m thick, were made in a coronal stereotaxic plane. Photographs of unstained sections were made with an enlarger prior to staining with cresyl violet. Serial sections were made through the entire extent of the implanted area. Electrode tip locations were determined by examination of each section for the most ventral extent of the glial scar. Since most brains were successfully sectioned in the plane of the implanted electrodes, a distinct outline of the concentric tips of the electrodes was easily observed. The anatomical site in which an electrode was located was designated by the nearest major nuclear or fiber

structure. For this report, data have been included only from tests of those stimulation sites verified to be located in the preoptic area or hypothalamus.

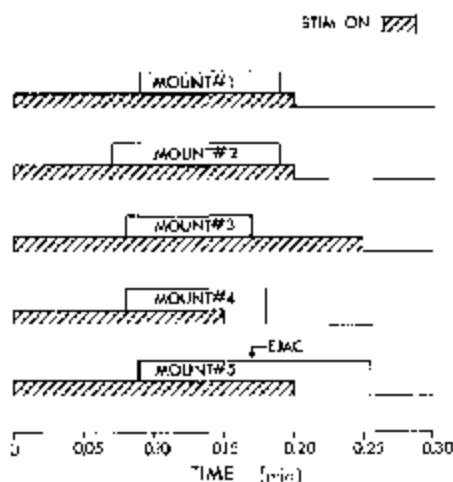
## Results

Mounting behavior was elicited during electrical stimulation of specific sites in the hypothalamus and preoptic area in each of 11 male rhesus monkeys in this study. Two basic types of sexual behavioral responses were produced by stimulation: (a) mounting with or without intromission and thrusting that was not associated with ejaculation and (b) a series of mounts with intromission and thrusting that led to ejaculation. These responses were observed during experiments in which social and hormonal variables were kept constant, i.e., tests with an estrogen-treated, ovariectomized female.

All responses classified as stimulus-induced mounts occurred with a latency of no longer than 0.2 min following the onset of a continuous train of pulses. Mount latency was relatively invariant in each subject but was, to some extent, determined by the distance separating the stimulated male from the receptive, estrogen-treated female. The duration of the induced mount was independent of the stimulus train duration. When the stimulus was prolonged, the male often would dismount; conversely, once intromission had occurred and pelvic thrusting was initiated, termination of the stimulus train would not cause the animal to dismount. Representative examples of the relationship between stimulus and mount duration are illustrated in figure 37.1. In rare instances, when the stimulus train continued for more than 30 sec, a second mount was occasionally observed prior to the end of stimulation. In a small number of subjects, sufficient measures of the duration of stimulus-produced mounts were obtained for comparison with spontaneously occurring sexual behavior. Mounts induced by stimulation of the medial preoptic area (MPO) or the DMH were significantly longer in duration than those observed in unstimulated, ejaculatory sequences (see table 37.1); however, this statistically significant increase was, in absolute terms, quite small for individual subjects.

### Location of Stimulus Sites

A total of 59 sites in the 11 males was histologically verified to be located within the hypothalamus and preoptic area. Among all subjects, 18 locations were effective for producing one of the two major types of sexual behavioral responses (see figure 37.2). Mounting behavior that did not culminate in ejaculation was elicited from the preoptic area in 11 of 19 sites that were generally confined to the dorsal aspect of the medial and lateral preoptic area and from a few sites imping-



**Figure 37.1**

Temporal relationships between mount and stimulus durations. Stimulation of the dorsomedial nucleus of the hypothalamus of a male rhesus monkey (NP 55) produced a series of mounts terminating in a mount with ejaculation. Mount latency was nearly constant. Mount duration was independent of stimulus length and stimulus offset. Stimulation parameters: 1.0 msec pulses at 50 Hz, 0.1 mA pulse current.

ing on the ventral aspects of the anterior commissure. Similar effects were obtained by stimulation of two of 11 tested loci in the DMH. Mounting that was associated with ejaculation was induced by stimulation of three within the DMH in three animals. The effective electrode placements in the DMH were tightly clustered in a portion of the nucleus that was slightly posterior to the midtuberal level of the hypothalamus within 1 mm of the midsagittal plane. Of the 9 tested sites that were located in the lateral hypothalamus, mounting accompanied by ejaculation was produced by stimulation of two electrode placement sites in two males. These sites were located medial to the optic tract at the level of the ventromedial nucleus. The remaining 41 preoptic and hypothalamic sites were ineffective in producing sexual behavioral responses; however, other social behaviors were obtained by stimulation of some of these locations. Stimulation of the anterior hypothalamic area (5 sites), ventromedial nucleus (5 sites) or posterior hypothalamus (10 sites) did not result in any sexual behavioral response in any of the tested males. In summary, mounting responses were obtained from only three areas: the preoptic area, the DMH and the lateral hypothalamus. The greatest percentages of effective sites were found in the preoptic area (57.9%) and the DMH (45.5%).

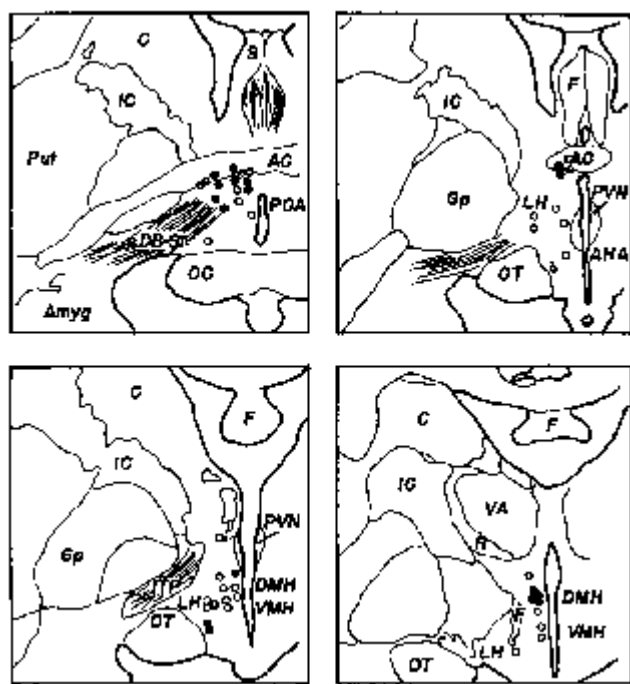
### Sexual Behavioral Measures Affected by Stimulation

The procedure for reliably inducing sexual responses involved the use of an iterative presentation of 10–15 sec duration trains of stimulation, at intervals of approximately 1 min. Using this approach, extensive tests

**Table 37.1**

Measures of male sexual behavior during preoptic/hypothalamic stimulation and during spontaneous mating behavior

Animal No.	Stim. Site	Mount Duration (Min $\pm$ S.E.M.)		$\bar{X}$ (Thrusts/Mount) $\pm$ S.E.M. <sup>§</sup>		$\bar{X}$ (Thrusts/Sec) $\pm$ S.E.M.		Stim. Duration (Min $\pm$ S.E.M.)	Mt. Latency	No Mounts	
		Non-stim.	Stim.	Non-stim.	Stim.	Non-stim.	Stim.			Non-stim.	Stim.
NP 54	DMH	0.06 $\pm$ 0.002	0.11 $\pm$ 0.004*	3.8 $\pm$ 0.12	9.3 $\pm$ 0.32*	1.2 $\pm$ 0.05	1.5 $\pm$ 0.04*	0.17 $\pm$ 0.004	0.06 $\pm$ 0.002	166	160
NP 55	DMH	0.07 $\pm$ 0.002	0.09 $\pm$ 0.002*	5.3 $\pm$ 0.15	7.8 $\pm$ 0.23*	1.3 $\pm$ 0.04	1.6 $\pm$ 0.05*	0.19 $\pm$ 0.004	0.09 $\pm$ 0.004	166	135
NP 85	MPO	0.04 $\pm$ 0.001	0.08 $\pm$ 0.003*	4.6 $\pm$ 0.11	7.4 $\pm$ 0.37*	1.9 $\pm$ 0.05	1.6 $\pm$ 0.07*	0.24 $\pm$ 0.004	0.16 $\pm$ 0.006	147	120
NP 47	MPO	0.05 $\pm$ 0.002	0.07 $\pm$ 0.005**	4.0 $\pm$ 0.19	4.5 $\pm$ 0.47	1.3 $\pm$ 0.06	1.0 $\pm$ 0.07***	0.17 $\pm$ 0.013	0.13 $\pm$ 0.012	89	27

<sup>§</sup>Includes only mounts accompanied by thrusts.\* $P < 0.001$ .\*\* $P < 0.005$ .\*\*\* $P < 0.01$ .**Figure 37.2**

Preoptic and hypothalamic stimulation sites. Closed circles represent locations of electrode placements from which mounting behavior was elicited. Open circles represent stimulation sites from which mounting was not elicited. The locations of the stimulation sites are shown in an estimated position relative to the closest stereotaxic plane of reference. Not shown are 12 tested hypothalamic sites, not associated with mounting, which were located posterior to the illustrated brain sections. Abbreviations: AC, anterior commissure; Amyg, amygdaloid complex; C, caudate nucleus; CP, cerebral peduncle; DB-SI, diagonal band-substantia innominata; DMH, dorsomedial nucleus; F, fornix; Gp, globus pallidus; H, fields of Forel; IC, internal capsule; ITP, inferior thalamic peduncle; LH, lateral hypothalamus; OC, optic chiasm; OT, optic tract; PH, posterior hypothalamus; POA, preoptic area; Put, putamen; R, reticular nucleus; VA, ventral anterior nucleus of the thalamus; VMH, ventromedial nucleus; ZI, zona incerta.

were performed on 4 males in order to examine in detail how elicited sexual responses compared to each animal's baseline of sexual activity. Stimulus parameters that produced consistent effects were selected in preliminary experiments.

In two males (NP 55, NP 54), stimulation of the DMH reliably produced ejaculatory sequences. The interval between spontaneous ejaculation and the beginning of the next sequence of mounts with intromission leading to ejaculation defined the refractory period. If stimulation was withheld, self-initiated mounting behavior was not exhibited by these males for 25–50 min during the post-ejaculation period. Stimulation was applied at each minute beginning 1 min following a spontaneous ejaculatory sequence. Immediately following ejaculation, DMH stimulation rarely resulted in sexual responses. During this portion of the refractory period, the stimulus usually produced alerting responses; occasionally, no manifest behavior was elicited. Within 5 min of ejaculation, mounts could be elicited; however, penile erections were not induced and intromission did not occur. Fifteen to twenty minutes following ejaculation, stimulation of the DMH produced mounts with intromission and thrusting. Upon repeated stimulation, additional mounts with intromission were induced, leading to an ejaculatory mount. If periodic stimulation was applied, this sequence of events was repeated. Thus, it was possible to decrease the interval (refractory period) between ejaculations (see figure 37.3). Consequently, for a standard test session, the number of ejaculations could be increased (see NP 54, figures 37.3 and 37.4). In comparison, although stimulation of MPO in two other males (NP 85, NP 47) consistently produced mounting with intromission, mount latency was longer than observed with stimulation of the DMH (see table 37.1). Furthermore, preoptic stimulation-produced mounting was insufficient to provoke an ejaculatory response.

In three of the four males from which measures were obtained the number of thrusts per mount was

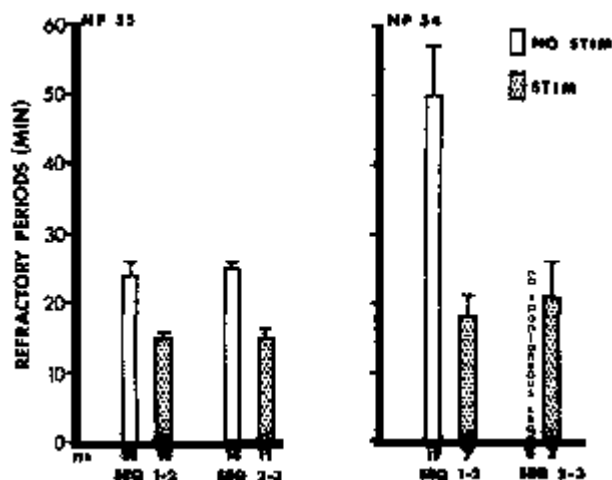


Figure 37.3

Average intervals in minutes ( $\pm$  standard error of the mean) between ejaculation and the beginning of the subsequent mount sequence leading to ejaculation within hour-long tests during which multiple ejaculations occurred. Clear bars indicate interval durations in tests during which brain stimulation was not applied. Stippled bars indicate interval durations between ejaculation and the occurrence of the first mount of an ejaculatory sequence elicited by DMH stimulation. For NP 54 (right half of the figure) no third sequence occurred without stimulation ( $n$  = number of refractory periods).

significantly increased with stimulation ( $P < 0.001$ , table 37.1). This increase is illustrated for successive ejaculatory sequences in the two animals with DMH electrode placements (figure 37.4). DMH stimulation also resulted in a significant increase in thrusting rate (mean number of thrusts per mount duration).

Although preoptic stimulation could increase the average number of thrusts per mount (NP 85, table 37.1), the thrusting rate was significantly decreased in both males bearing MPO electrodes. This may account for the absence of ejaculatory responses to MPO stimulation. For example, during stimulation of the preoptic area in NP 85 mounting and intromission would occur. Despite repeated stimulated mounts, ejaculation did not occur even after a greater number of mounts than observed in spontaneous ejaculatory sequences. Ejaculations for NP 85 ordinarily occurred at the end of a mount series ranging from 2 to 10 mounts. In three separate stimulation sessions, ejaculation was not observed even after as many as 41 mounts ( $\bar{X} = 34.7 \pm 4.9$ ) had been elicited. In one animal (NP 47), medial preoptic stimulation did not result in an increased number of thrusts per mount. This may be accounted for, in part, by the fact that only 31.8% of the elicited mounts were accompanied by intromission or pelvic thrusting when stimulation was delivered during the post-ejaculatory period. In this male, attempts were made to facilitate sexual performance by applying preoptic stimulation during self-initiated mounts. This resulted in an increase in the mean number of thrusts

per mount (13.5,  $n = 59$ ) over that observed during spontaneous mounts (4.01,  $n = 89$ ). Occasionally, under these circumstances, ejaculation would occur during a self-initiated mount during which stimulation was delivered to the medial preoptic area.

#### Effects of Female Hormonal Status

Since the hormonal status of the female is an important variable in normal copulatory activity in rhesus monkeys, the effects of manipulation of estrogen in the female partner on elicited male sexual behavior was tested with a single animal (NP 55). The male was paired in tests with each of three ovariectomized females that were given estradiol benzoate by injection. Stimulation was delivered following a spontaneous ejaculatory sequence or, if no sexual activity occurred, 5 min after the female was introduced to the male. Following these tests, estrogen treatment was terminated. After a minimum of 5 weeks, during which regularly scheduled measures of spontaneous sexual performances were made, stimulation effects were re-tested. Results are summarized in table 37.2. The withdrawal of estrogen diminished spontaneous sexual activity and eliminated ejaculation. Similarly, when tested with untreated females, the stimulated male mounted less often and exhibited fewer thrusts per session than when paired with estrogen-treated females. Furthermore, except for one test, ejaculatory sequences were not produced by stimulation. Thus, for all measures, the sexual responses of the male to stimulation paralleled changes in spontaneous sexual behavior associated with the hormonal condition of the female.

#### Other Behavioral Responses

A number of other manifest responses were observed upon stimulation of both sex behavior-producing sites and other hypothalamic and preoptic areas. One such response of potential functional significance with regard to sexual responses produced by brain stimulation was penile erection. This response could be observed in the restrained animal as well as when the same male was stimulated in the presence of a female. When the data from the 11 males in this study were analyzed for an overall correlation between penile erection and mounting responses obtained from stimulation of any locus within the preoptic area or hypothalamus, no significant relationship was found between the two responses for 57 hypothalamic or preoptic sites (Chi square = 0.17,  $df = 1$ ). (For two of the 59 hypothalamic or preoptic electrode placements in the total sample, no observations were made for elicited penile erections.)

When 19 of the 57 sites were stimulated, penile erections were elicited in the restrained subjects, but no mounting responses were observed during stimulation

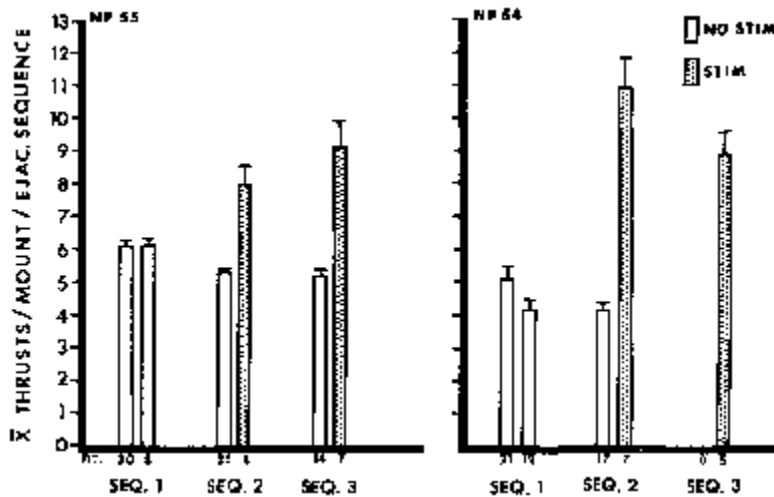


Figure 37.4

Average number of thrusts per mount ( $\pm$  standard error of the mean) within a series terminating in ejaculation in two males. Each pair of bars represents data averaged from the first, second or third ejaculatory sequences. Stimulation (stippled bars) was applied only for the second and third sequences; the right-hand bar of each pair represents measures for ejaculatory sequences during stimulation sessions. Clear bars indicate that no stimulation was applied during the ejaculatory sequence. The left-hand bar of each pair was derived from observations during non-stimulation sessions ( $n$  = number of ejaculatory sequences).

Table 37.2

Average number of mounts, thrusts and ejaculations which occurred in non-stimulation and stimulation sessions when one male (NP 55, DMH site) was paired with ovariectomized females under two hormonal conditions

Hormonal Status of Female*	Non-Stimulation Sessions				Stimulation Sessions**				
	Mounts	Thrusts	Ejac.	n (Sess.)	Mounts	Thrusts	Ejac.	$\bar{X}$ (No. Stim.)	n (Sess.)
With estrogen	7.5	37.5	1.1	11	12.4	67.5	1.6	17.0	8
Without estrogen	0.3	0.0	0.0	11	5.5	10.2	0.1	23.7	10

\*Three females were paired separately with the male.

\*\*Stimulation was applied to the dorsomedial nucleus of the hypothalamus.

in the social test setting. When stimulation was applied to those sites while the male was spontaneously mounting, a dismount occurred shortly after the onset of the stimulus train. The ejaculatory sequence was disrupted if the stimulus was repeated during subsequent mount attempts. This effect was obtained in a portion of the sites stimulated in each anatomical area investigated (see table 37.3); the sole exception was the lateral preoptic area. Eleven of these sites (58%) were found in areas from which mounting responses could not be elicited, i.e., the anterior hypothalamus ( $n = 5$ ), the ventromedial nucleus ( $n = 2$ ) and the posterior hypothalamus ( $n = 4$ ).

Among 17 sites from which mounting was elicited, 53% were effective for producing penile erections (see table 37.3). These 9 sites, which were confined to the preoptic area and the DMH, accounted for 32% of all loci from which the erection responses were obtained. Penile erections were not produced by stimulation of either of the two sites in the lateral hypothalamus associated with mounting responses. Combined responses

of penile erection and mounting were obtained by stimulation of three sites in the medial preoptic area, two in the DMH and 4 placements located in the lateral preoptic area or ventral aspects of the anterior commissure. Therefore, although as a general rule, sexual behavioral responses were not correlated with penile responses to stimulation of the hypothalamus, it appears at least for the lateral preoptic area that a correlation may exist between the two effects.

Stimulation of specific loci within the preoptic area and the hypothalamus produced some form of aggressive behavior in all subjects. These responses could generally be classified in one of three categories: attack, non-contact aggression or aggressive display (1). Attack responses were characterized by a rapid pursuit by the stimulated male of the other animal(s) in the cage, followed by attempts to restrain and bite the victim. Non-contact aggression responses were less violent, involving vigorous chasing and lunging throughout the duration of the stimulus. Aggressive displays involved facial gestures, non-directed stares and cage

**Table 37.3**

Distribution of hypothalamic and preoptic stimulation sites associated or not associated with mounting and the percentage of those sites from which penile erection and/or agonistic behaviors could be elicited

Anatomical Area		Number of Sites Associated with Mounting	% "Mount Sites" Associated with Other Behaviors			Number of Not Sites Associated with Mounting	% "Non-Mount Sites" Associated with Other Behaviors		
			Erection Only	Agonistic Only	Erection and Agonistic		Erection Only	Agonistic Only	Erection and Agonistic
POA	LPO	3	0	0	100	3	0	0	0
	MPO	5	0	0	60	4	50	25	0
	AC/NAC	3	0	33.3	33.3	1	—*	0	0
MBH	AHA	0	0	0	0	5	60	0	40
	DMH	5	20	40*	20	6	16.7	33	50
	VMH	0	0	0	0	5	0	60	40
	LH	2	0	50	0	7	0	57	28.6
	PH	0	0	0	0	10	40	30	0
Total		18				41			

Abbreviations: POA, preoptic area; LPO, lateral preoptic area; MPO, medial preoptic area; AC/NAC, anterior commissure/nucleus of the anterior commissure; AHA, anterior hypothalamus; MBH, medial-basal hypothalamus; DMH, dorsomedial nucleus; VMH, ventromedial nucleus; LH, lateral hypothalamus; PH, posterior hypothalamus.

\*One site not tested for elicited penile erection.

shaking behavior, but no attempts to chase or punish other members of the group.

Among the 18 sites that were effective for producing mounting behavior, attack responses were elicited from 6 electrode placements and non-contact aggression was obtained from 5 stimulus sites (see table 37.3). Except for a single locus in the lateral preoptic area, the stimulus intensity for producing mounting responses was lower than that required for eliciting attack. Non-contact aggression was obtained during stimulation of two sites in the preoptic area and three sites in the DMH, which were also effective for eliciting mounting responses. Among the 41 electrode placements that were ineffective for producing mounting, attack responses were induced by stimulation of 16 locations and display responses were elicited from 5 sites. Of particular interest was the number of stimulus sites from which aggression alone was elicited, that were located adjacent to other sites associated with mounting responses in the preoptic area, DMH and lateral hypothalamus. Of 6 such sites in the DMH, 5 were associated with attack or display responses. In the lateral hypothalamus, where two locations were found for eliciting mounting responses, 6 of the 7 other tested placements were effective for eliciting attack. Stimulation of 8 sites in the preoptic area did not produce mounting responses; of these, only one was effective for eliciting attack.

Specific illustrations of the relationship between aggressive and sexual behavioral responses can be drawn from some of the subjects with electrodes implanted in the DMH or preoptic area. For example, stimulation of the DMH in NP 55, at higher current levels than

required for a sexual response, would elicit a threat behavior directed toward the female. The DMH electrode site in NP 55 was located slightly ventral to that for NP 54, placing it closer to the ventromedial nucleus of the hypothalamus which we have found, in previous studies (28), to be a site from which attack can be produced by stimulation. Therefore, the sexual responses obtained by stimulation of the DMH apparently were not the result of spread of current involving nuclear structures outside that area, but involved a relatively restricted area. In contrast, stimulation of the lateral preoptic area produced both sexual and aggressive behavior often with the same stimulus parameters (28, 30). In three subjects, stimulation of the lateral preoptic area sites associated with mounting responses elicited biting attack responses concurrent with mounting behavior when the stimulus intensity was increased. For example, immediately after stimulus onset the mate would approach the female and mount; he would then reach forward, grasp the female and bite the back of her shoulders or neck.

## Discussion

The evidence provided by the present study suggests the existence of mechanisms in several loci within the preoptic area and hypothalamus that are related to the regulation and expression of sexual behavior in male rhesus monkeys. Mounting responses were obtained by electrical stimulation of the preoptic area, lateral hypothalamus and dorsomedial nucleus of the hypothalamus and ejaculatory sequences were produced by stimulation of the latter two sites. Since the

locations of the effective sites for mounting responses appeared to be defined by clusters that were surrounded by stimulus sites associated with agonistic or other behavioral responses, the findings suggest that stimulation of specific loci can elicit specific behavioral effects. Further support is found for this argument in the existence of different thresholds for contrasting behavioral effects of stimulation of given anatomical sites. In addition, in some animals both aggressive and sexual behavioral responses were obtained by stimulation of different loci. Thus, it appears that the nature of the behavioral effects of hypothalamic and preoptic stimulation cannot simply be explained in terms of non-specific arousal mechanisms.

A considerable body of evidence has been developed implicating the medial preoptic/anterior hypothalamic area in the regulation of sexual behavior. In general, lesions in this region have been found to disrupt sexual performance in males of different species (9, 12–14, 18). Male rhesus monkeys with lesions of the medial preoptic/anterior hypothalamic area either cease copulating with females or are greatly impaired in their sexual abilities; however, these same males masturbate to ejaculation when socially isolated in their home cages (36). The results of the present study are consistent with previous reports of the effects of electrical stimulation of the preoptic area on male sexual behavior (23, 39). We were unable, with preoptic stimulation, to produce ejaculation although the stimulus was quite effective for inducing mounts. These findings indicate that the preoptic area may be involved in controlling the initiation of copulation. Thus, the effects obtained by stimulation of medial and lateral preoptic area in male rhesus monkeys may be comparable to the observation that medial preoptic area stimulation facilitates sexual activity in male rats (22).

Lesions and electrical stimulation of the medial forebrain bundle at various levels throughout the lateral hypothalamus influence sexual behavior in male rats (4, 6, 23). In a small percentage of lateral hypothalamic sites tested for sexual responses in the present study, ejaculatory sequences could be induced by periodic stimulation. These results may be compared to those reported for stimulation of comparable sites in the male rat (6).

The new finding reported here is that electrical stimulation of the DMH will induce the complete copulatory pattern of male rhesus monkeys. This area is quite small and difficult to destroy without involving the paraventricular and the ventromedial nuclei. We can find no report of a systematic investigation of the effects of lesions of this small area on male sexual behavior. However, Heimer and Larsson (14) reported no alteration in male sexual behavior of rats when lesions

were placed in the dorsal region of the middle hypothalamus.

Since arousal effects are a consideration in the evaluation of behavioral responses to brain stimulation (16, 37), one must account for non-specific stimulus effects. Painful electrical shocks, delivered to the tail or the skin of the flanks of rats, will induce copulatory behavior in sexually experienced (2, 7, 35), inexperienced (5), juvenile (10) male rats and androgen-treated gonadectomized female rats (8, 17). Malsbury and Pfaff (23) have distinguished the effects of peripheral stimulation from those induced by medial preoptic stimulation in terms of Beach and Jordan's (3) formulation of two mechanisms underlying male rat sexual behavior by proposing that medial preoptic area stimulation facilitates components of the "copulatory-ejaculatory mechanisms" whereas peripheral stimulation (e.g., tail pinch or shock) or stimulation of the posterior hypothalamus (4), exerts influences on sexual behavior through the "arousal mechanism."

The present set of experiments provides three lines of evidence which indicate that the responses to stimulation of specific regions of the hypothalamus and preoptic area are the result of the influence of a "copulatory-ejaculatory mechanism" rather than the consequence of the activation of an "arousal mechanism" that directs the animal to orient toward the female. First, a number of discrete sites, separate from those from which mounting responses were obtained, were found to be effective for eliciting an obvious arousal that was manifested as a form of agonistic behavior. This response involved a direct approach toward the female, however, no component of sexual behavior was observed during the course of this response. As noted above, only within the lateral preoptic area were combined mounting and aggressive responses obtained by the use of the same stimulus intensity. Similar combined behavioral effects have been reported for lateral preoptic stimulation in other species (24, 39). The second line of evidence derives from the finding that stimulation of the medial preoptic area or the DMH produced specific changes in components of copulatory behavior. These quantified effects represented more than a simple increase in approach toward and contact with the female. Finally, the relationship of the elicited behavior to spontaneously occurring sexual activity was indicated by the sensitivity of the mounting response elicited by DMH stimulation to the hormonal status of the female and to the social status of the stimulated male (28).

Genital responses elicited in restrained animals may represent a component of a more complete behavioral response than would be seen if the animals were tested unrestrained with a receptive female. With the

exception of our work, the findings of previous research on non-human primates have been confined to the effects that can be observed by electrical stimulation of the monkeys while they were seated in chairs (20, 34). Our present findings emphasize the importance of careful interpretation of results of such studies. In particular, penile erections cannot be presumed to be indicative of a mechanism directly related to copulation. On the other hand, when stimulation produces ejaculation in the restrained animal (30, 33), one may more readily accept this response pattern as being pertinent to behavior. However, none of the animals involved in the present study exhibited ejaculation as a result of stimulation either at the time of implantation or in subsequent tests with the animals socially isolated or restrained. These findings re-emphasize the importance of tests performed in the presence of appropriate goal objects in order to characterize adequately the behavioral response and to quantify stimulus effects through specific measures of sexual performance. Although the artifactual nature of electrical stimulation has been recognized (37), the advantages of this approach, particularly with regard to small nuclear areas of the hypothalamus, are evident in one of the findings of the present study implicating the dorsomedial nucleus of the hypothalamus in the regulation of male sexual behavior in rhesus monkeys.

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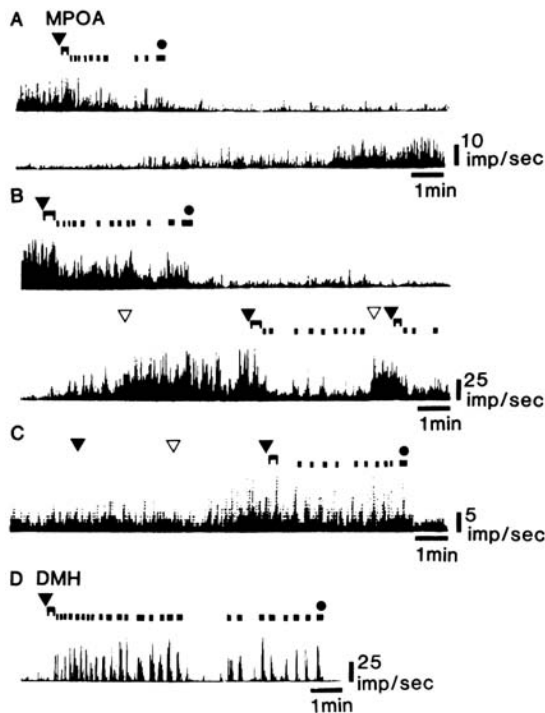
The medial preoptic area (MPOA) appears to be involved in the expression of sexual behavior. To date, a few reports about central control mechanisms of sexual behavior in monkeys have discussed the role of the MPOA relative to changes in components of sexual behavior. Electrical MPOA stimulation evoked penile erection (2, 8), ejaculation (7), and mounting and thrusting (5), and lesions reduced mating activity (10). Recently, we succeeded in recording stable single neuron activity in the MPOA and the dorsomedial hypothalamic nucleus (DMH) of partially restrained monkeys during actual sexual performance, and found close correlation between neuronal activity in these centers, sexual arousal, and behavioral acts.

Three rhesus monkeys, *Macaca mulatta* (two males 6 kg, one female 5 kg), were used. Under anesthesia, adaptors for Evarts-type positioners were attached to the skulls of the male monkeys. After 2 weeks recovery, single neuron discharges from the MPOA and the DMH were recorded through glass-coated elgiloy electrodes (12) during sexual behavior. Electrode placement was determined stereotactically (11) and radiographically and later verified histologically. Restraining apparatus was a fixed primate chair for the male which faced a mobile chair for the female. The male monkey was placed in a position convenient for mounting by lowering the floor of the chair. The chairs were initially set at 1 m apart with an interposed shutter. After the shutter was opened by the experimenter, the male monkey was able to gain access to the female by pressing a bar. Each bar press advanced the monkey's chair approximately 5 cm thus requiring 20 bar presses to bring the female close enough to perform the sexual act. This operant paradigm was used to assess the degree of sexual desire (3, 4) and was regarded as a signal of initiation of sexual behavior. Mating acts (including manual contact, mounting, intromission, and thrusting) followed access to the female monkey. These were repeated until ejaculation terminated sexual behavior. This trial sequence could be repeated 2 or 3 times within 6 h of each day in the monkey's mating season. Bar press performance

depended on previous sexual activity for each day. For instance, during the refractory period following ejaculation, bar pressing did not occur, or was initiated with a longer latency to the first bar press, and was discontinuous compared to the first trial of the day or to a trial delayed for 30 min after an ejaculation.

Examples of single neuron activity in the MPOA, recorded during sexual encounters, are shown in figure 38.1A, B and C. In many MPOA neurons, the firing rate was high even before the commencement of sexual behavior (upper tracing in figure 38.1A and B). This possibly reflected anticipation of a sexual encounter, based on previous trials, or sensory stimulation by female odors (3), or both. Generally, MPOA neuronal activity began to decrease upon acquisition of a female and continued to decrease throughout the mating activity. It reached its lowest level immediately after ejaculation, and remained low, but gradually recovered in 25 min (lower tracing in figure 38.1A and B). Both animals usually engaged in grooming behavior during the refractory period. The recovery of neuronal activity appeared to be highly correlated with recovery from the refractory period and seemed to indicate sexual readiness. This type of pattern was recorded from 17 of 21 MPOA neurons tested during sexual sequences.

Various manipulations altered the time course of MPOA responses. Separating the monkeys during the late phase of recovery following ejaculation elicited increased neuronal activity (open triangle, left portion of lower tracing in figure 38.1B). Opening the shutter for the second trial elicited activity change similar to that observed during the first trial. Separation before ejaculation increased neuronal activity (right portion of lower tracing in figure 38.1B). Neuronal activity increase due to separation, never occurred immediately after ejaculation or during the early phase of the refractory period. The recording in figure 38.1C was begun during the refractory-recovery stage following ejaculation and separation after the first trial. Opening the shutter caused no change in either the neuronal activity or behavior of the animal. After closing the shutter, neuronal activity increased with a small delay. In



**Figure 38.1**

Rate meter plots of MPOA (*A, B, C*, different neurons), and DMH (*D*) activity during sexual behavior, ▼, opening of shutter interposed between male and female; ■ bar presses (about 20), ■ mating sequence (manual contact, mounting, intromission, thrusting). Length of each mark indicates duration of mating act. ●, ejaculation; ▽, separation of animals and shutter closing. ▽ in *C* indicates shutter closing only, since male and female were already separated. *A* and *B*: continuous recording from upper to lower of each pair of traces. Neuronal activity was high before commencement of sexual behavior and decreased after acquisition of a female. Activity became lowest after ejaculation and gradually recovered. *B*: during gradual recovery of neuronal activity, first separation of animals caused sharp increase. Second shutter opening elicited bar pressing. During performance of sexual behavior MPOA activity decreased again. Separation before ejaculation increased neuronal activity. Third shutter opening elicited decrease similar to that seen in second trial. *C*: during refractory period, after first ejaculation (not shown), male did not initiate sexual behavior during low neuronal activity level (first half of record), but did so during high level of neuronal activity (second half of record). *D*: synchronized increase in DMH neuron activity during each mating act.

contrast, when the shutter was opened again during a period of high neuronal activity, the male monkey engaged in complete sexual behavior.

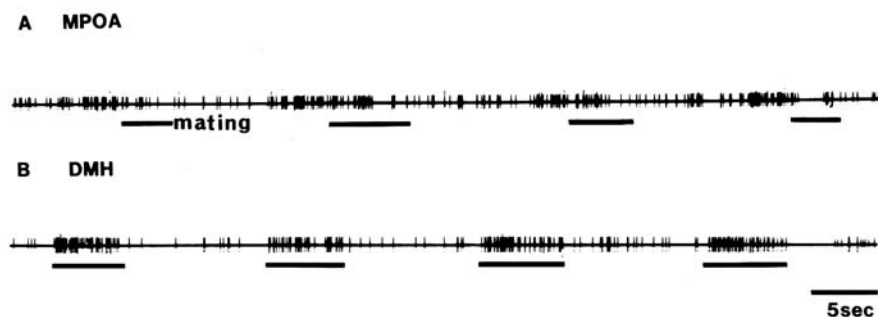
Comparison of neuronal activity with behavior patterns suggest that MPOA neurons reflect the animal's sexual arousal, initiation and completion of sexual behavior. Lesions of the medial preoptic-anterior hypothalamus were reported to not disturb the animal's masturbatory activity but impair sexual behavior with a female monkey (10). MPOA neurons of the male monkey may thus integrate information concerning the presence of a receptive female through visual and olfactory cues, and direct the male monkey to initiate copulation.

Some MPOA neurons (8 of the 17 which responded) also increased in activity prior to each mating act before actual contact with the female. Penile erection correlated with neuronal activity increase before mounting (figure 38.2A). This suggests that, in addition to sexual arousal these neurons facilitate the parasympathetic activity responsible for initiation of penile erection. This agrees with the MPOA's well known relation to parasympathetic functions (6). After intromission, however, erection is maintained by peripheral reflex at the spinal level. The increase in sympathetic activity which is also well known to be required for ejaculation again indicates decreased MPOA activity.

Activity increase monitored in DMH neurons was closely associated with the occurrence of mounting, intromission and thrusting during mating sequences (figure 38.2B). Discharges of these neurons were relatively few during all other phases of sexual behavior (figure 38.1D). This pattern was observed in 9 of 15 neurons tested in the same area. In a recent report (5), electrical stimulation of the MPOA or the DMH of a freely moving male monkey in the presence of a receptive female produced different sexual behavior. Stimulation of the MPOA as well as the DMH produced mounts of longer duration and increased the number of thrusts per mount. The thrusting rate was decreased by MPOA stimulation and increased by DMH stimulation. Ejaculation was evoked by DMH stimulation, but not by MPOA stimulation. Our electrophysiological findings in the DMH thus concur with previous behavioral data which suggest that this region is involved in copulation, i.e., the actual performance of sexual behavior, but is not necessarily related to sexual arousal. In addition, DMH activity may be involved in the autonomic mechanism related to ejaculation after intromission, since the DMH is one of sites which have direct connections to the preganglionic nuclei of the sympathetic nervous system (9).

These neuronal activity patterns, which have been observed here for the first time, seem to correlate specifically with sexual behavior. The activity patterns of these MPOA neurons were not affected by food on the female chair in place of the female, although the monkey did press the bar to obtain the food. Also, DMH neuron activity did not correspond to random motor activity, but exhibited its specific pattern only in relation to sexual acts.

It has been proposed that male sexual behavior in the rat is controlled by a dual mechanism (1). The first part controls the initiation of sexual arousal, and the second controls performance of copulatory behavior such as mounting, intromission, thrusting and ejaculation. The present study indicates that this dual mechanism may be regulated from the MPOA and DMH, but does not exclude other regulatory sites. The MPOA reflects sex-

**Figure 38.2**

Neuron impulses in MPOA (A) and DMH (B) at fast sweep. ■, mating acts (manual contact, mounting, intromission and thrusting). A: neuronal activity increased prior to mating act. B: synchronized increase in neuronal activity during each mating act.

ual arousal, central initiation of behavior, while the DMH actual performance of sexual behavior.

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Several *morphological* sex differences have been identified in and around the medial preoptic area of the rodent brain (Raisman and Field, '73; Greenough et al., '77; Gorski et al., '78, '80). The most striking consists of a dense cluster of darkly staining cells embedded within the medial preoptic nucleus (MPN: Gurdjian, '27) of the rat. This dense cell group, the *sexually dimorphic nucleus of the preoptic area* (Gorski et al., '80), is several times larger in males than in females, although the functional significance of this difference is unknown. The medial preoptic area as a whole has been implicated in a variety of physiological mechanisms and behavioral responses including adenyohypophyseal regulation (Szentágothai, '68), especially gonadotrophin release (Gorski, '68; Wiegand and Terasawa, '82), thermo-regulation (Squires and Jacobsen, '68; Day et al., '79), hypovolemic thirst (Swanson et al., '78; Swanson and Mogenson, '81), maternal behavior (Jacobsen et al., '80), and sex behavior (Gorski, '74; Clemens and Gladue, '79). Little is known about the specific role of the MPN and sexually dimorphic nucleus in these functions. It has been suspected for some time, however, that the region of the MPN is involved especially in reproductive behavior, since bilateral lesions that include at least part of the MPN disrupt or eliminate copulatory behavior (Heimer and Larsson, '66; Ginton and Merari, '77; Christensen et al., '77; Nance et al., '77; Ryan and Frankel, '78; Hansen et al., '82), while electrical stimulation in the vicinity of the MPN produces a significant enhancement of this behavior (Van Dis and Larsson, '70; Malsbury, '71; Napoli et al., '72; Merari and Ginton, '75).

Monoaminergic neurotransmitter systems have been implicated in the control of reproductive behavior, although the specific pathways involved have not been established. A growing body of evidence indicates that serotonin plays an important role in the control of two sexually dimorphic reproductive functions—gonadotrophin release (Waloch et al., '81) and mating behavior (Crowley and Zemlan, '81). Foreman and Moss ('78) and Ward et al. ('75) have reported that serotonin may influence female mating behavior at the

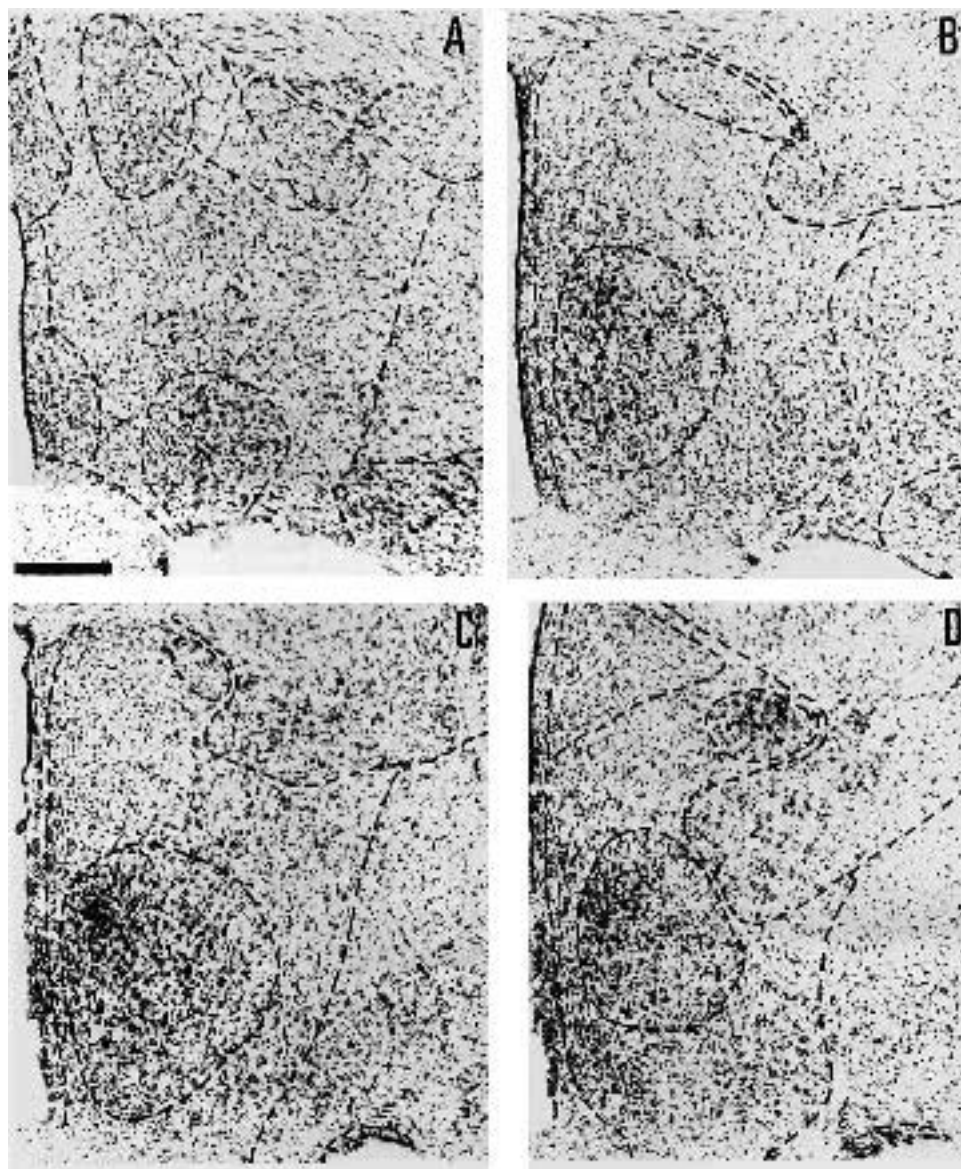
level of the medial preoptic area, although no specific cell groups within this area were implicated.

Serotonin has been found in the medial preoptic area by using biochemical (Saavedra et al., '74), histofluorescence (Fuxe, '65), autoradiographic (Chan-Palay, '77; Descarries and Beaudet, '78; Parent et al., '81), and immunohistochemical (Steinbusch, '81) methods. However, these studies provide little information about the differential distribution of serotonergic fibers, and comparative data about the relative levels of serotonin in the MPN of males and females is not available. Using biochemical methods, sex differences in serotonin content have been shown for whole brain (Kato, '60; Ladowsky and Gaziri, '70) and for hypothalamus (Gladue et al., '77). In addition, females reportedly have significantly higher levels of monoamine oxidase activity in the hypothalamus than do males (Kamberi and Kobayoshi, '70; Rosecrans and Schechter, '72).

To increase our understanding of the intrinsic organization of the sexually dimorphic component of the MPN and its possible relationship to serotonergic transmitter systems, we have undertaken an immunohistochemical study of the distribution of serotonin-immunoreactive fibers in and around the MPN of male and female rats. The accuracy of mapping was greatly facilitated by the use of a fluorescent Nissl counterstain (Schmued et al., '82), which made possible a detailed correlation of the distribution of immunoreactive fibers with cytoarchitectonic details in the same tissue section.

#### Materials and Methods

Five adult male (350–400 gm) and five adult female (300–350 gm) Sprague-Dawley rats were used in this study. The animals were maintained on 14:10-hour lighting with food and water ad libitum. The females were perfused on the afternoon of estrus, as verified by vaginal smear. Groups of animals containing both males and females were anesthetized with chloropent and perfused with a variation of the Bérød-Hartman method (Swanson et al., '83). The brains were removed



**Figure 39.1**

Photomicrographs of thionin-stained, 30- $\mu$ m-thick frontal sections through the medial preoptic area and adjacent regions in the male rat, arranged from rostral (A) to caudal (D). Outline drawings of these micrographs appear in figure 39.2. Scale = 300  $\mu$ m.

immediately and postfixed overnight at 4°C with 10% formalin in acetate buffer at pH 9.0, containing 15% sucrose. The following day, the brains were washed for 5–10 minutes in cold distilled water, frozen with dry ice, and 30- $\mu$ m-thick sections were cut on a sliding microtome. Every other section through the MPN was collected and processed for immunofluorescence as described previously (Sawchenko and Swanson, '81), using an antiserum directed against serotonin at a dilution of 1:1,000. The antiserum was a generous gift from Dr. Harry Steinbusch, who has published details about its preparation and specificity (Steinbusch et al., '78; Steinbusch, '81). The alternate sections were mounted

and stained with thionin for use as a Nissl-stained reference series. Following localization of the primary antiserum with affinity-purified goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC; Hartman, '73), the sections were mounted onto gelatin-coated slides from potassium phosphate-buffered saline and counterstained with ethidium bromide (Schmued et al., '82). The slides were coverslipped with buffered glycerol (Hartman, '73) and examined with a Zeiss Universal fluorescence microscope. The fluorescein-labeled secondary antibody was visualized with a filter system that provides blue excitation wavelengths (440–490 nm), while the ethidium bromide Nissl counter-

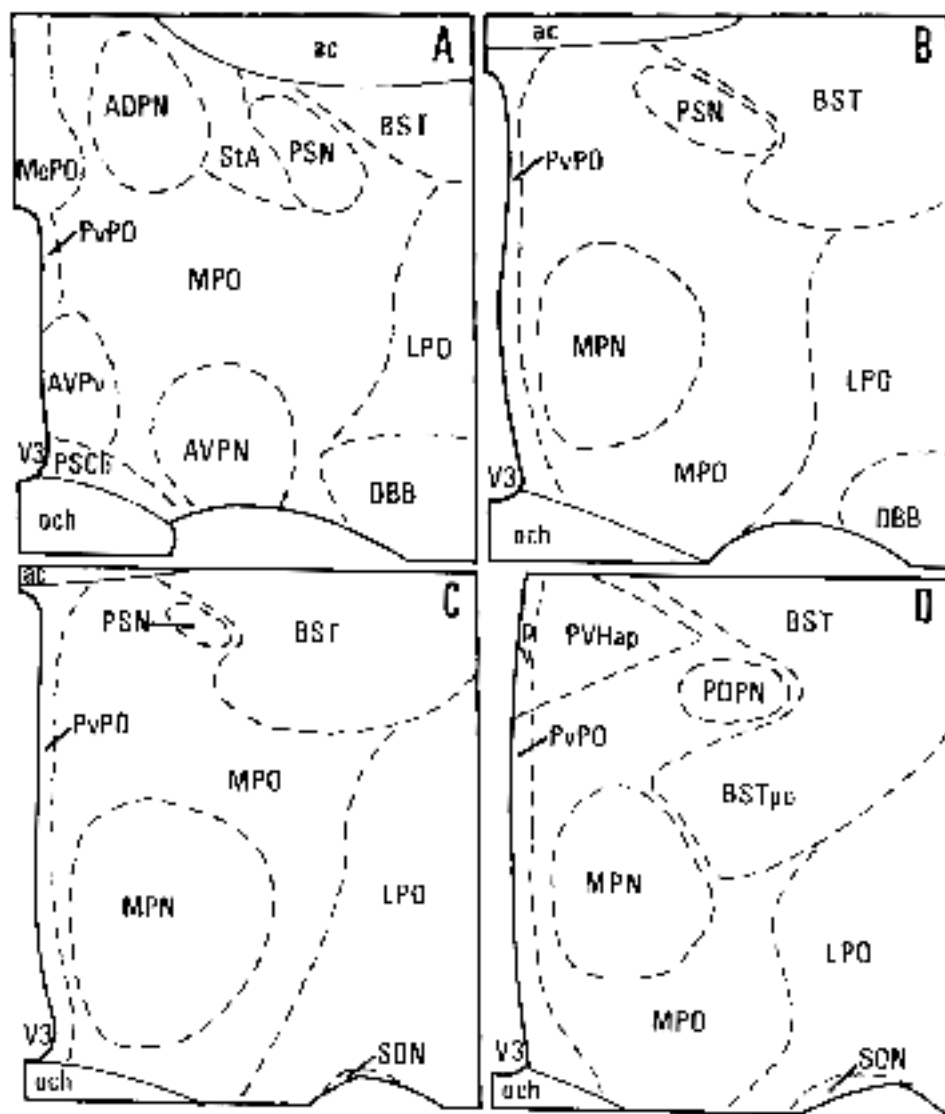


Figure 39.2

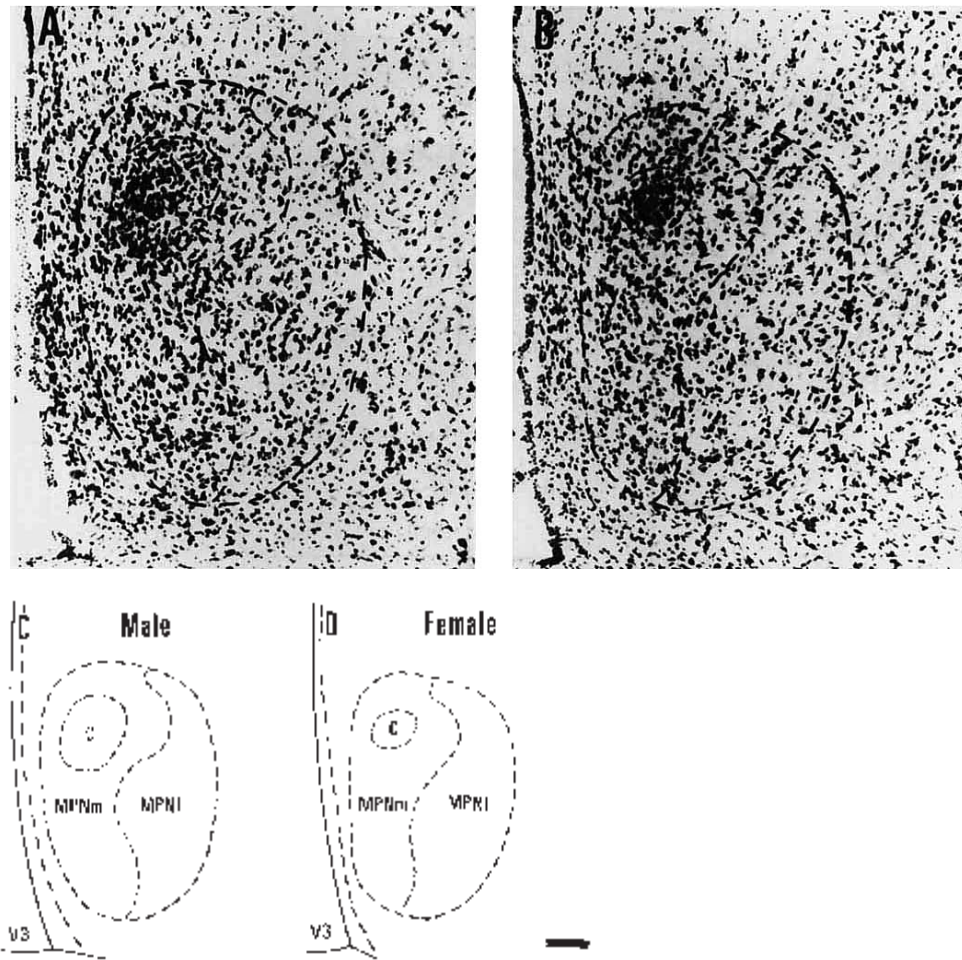
Drawings to indicate the boundaries of cell groups and fiber tracts in and adjacent to the medial preoptic area as illustrated in figure 39.1.

stain was visualized with a second filter system that provides green excitation wavelengths (510–569 nm). The distribution of serotonin-immunoreactive fibers was mapped without knowledge of the sex of the animal onto camera lucida tracings from the adjacent Nissl-stained reference series. The distribution of stained fibers was correlated with cytoarchitectonic features of the MPN identified in both the ethidium bromide-counterstained series and the thionin-stained adjacent series. To quantify the density of immunostaining, fiber counts were made at 10- $\mu$ m intervals (in 100- $\mu$ m<sup>2</sup> squares) along a defined traverse across the MPN, at a magnification of  $\times 500$ . Fiber density profiles were constructed for each animal and compared.

## Results

### Normal Morphology of the Medial Preoptic Area

Before describing the results of our immunohistochemical studies, it is necessary to consider the normal morphology of the preoptic region in some detail since its parcellation is a source of great confusion. There is general agreement that the preoptic region can be divided into periventricular, medial, and lateral zones, or areas (e.g., Gurdjian, '27; Swanson, '76; Bleier et al., '79), although opinions differ about further subdivisions of the medial preoptic area. This area clearly extends throughout the length of the preoptic region and is limited dorsally by the anterior commissure, ventrally by the optic chiasm, medially by the



**Figure 39.3**

Photomicrographs of thionin-stained sections through the MPN at the level of the MPNc to show the boundaries of the three subdivisions of the nucleus in the male (*A*) and female (*B*) rat. *C*, *D*. Outline drawings of the photomicrographs in *A* and *B*, respectively. Abbreviations for parts of the MPN: MPNl, lateral subdivision; MPNm, medial subdivision; c, central subdivision. The level of photomicrographs *A* and *B* correspond roughly to that in figure 39.5B and 39.5F, respectively. Scale = 150  $\mu$ m.

periventricular preoptic nucleus, and laterally by the fiber-rich lateral preoptic area. Caudally, the medial preoptic area comes to lie dorsal to the less-cell-dense anterior hypothalamic area, which replaces it at more posterior levels (Saper et al., '78). Rostrally, the medial preoptic area extends to the level of the lamina terminalis. In the following description, which is based on the examination of serial Nissl-stained sections cut in the three standard planes of section, we shall concentrate on the MPN itself, although it will be necessary to consider briefly the cell groups that surround it as well.

The medial preoptic area can be viewed as an undifferentiated hypothalamic gray in which several cellular condensations, or nuclei, are embedded. The large centrally placed MPN (Gurdjian, '27: his figure 39.1) extends nearly the length of the medial preoptic area (figures 39.1, 39.2). It is oval-shaped in frontal sections, lies at the level of the decussation of the anterior

commissure, and extends for approximately 1,100  $\mu$ m in the rostrocaudal axis.

The medial border of the nucleus is separated from the periventricular preoptic nucleus by a relatively cell-sparse zone, while its lateral border is somewhat more difficult to define in Nissl-stained material. The cells near the periphery of the MPN tend to be fusiform in shape and are primarily oriented tangentially to the circumference of the nucleus (figure 39.3). The MPN as described here corresponds, in general, to the "nucleus preopticus principalis" of Loo ('31) and Young ('36). Bleier et al. ('79, '82) designate this nucleus as the "anterior hypothalamic nucleus." It should also be noted that the term "medial preoptic nucleus" has been used by many workers in virtual synonymy with the medial preoptic area (e.g., LeGros Clark, '38; König and Klippel, '63; Nauta and Haymaker, '69; Palkovits and Záborszky, '79).

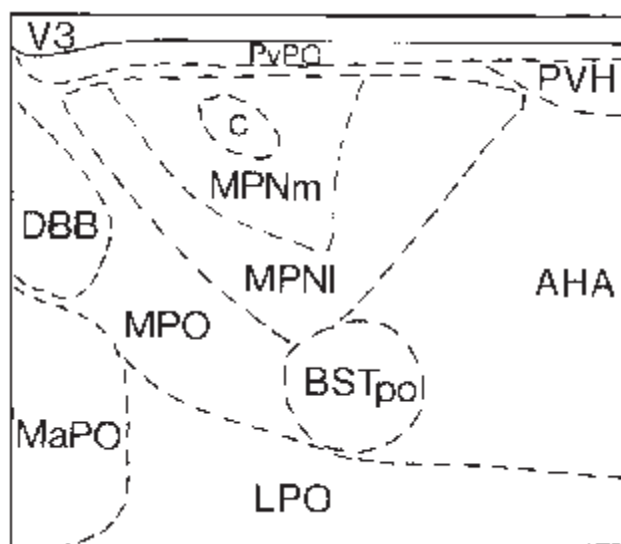
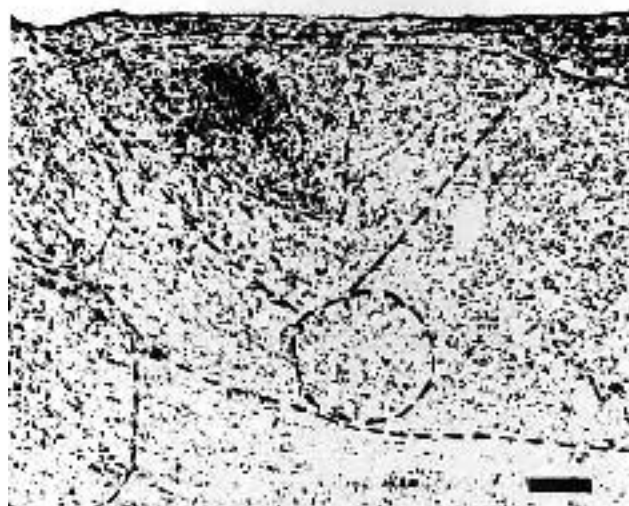
Four smaller nuclei can also be distinguished in the medial preoptic area. At rostral levels, a dorsal cell group, which we shall call the *anterodorsal preoptic nucleus* (ADPN, figure 39.1A), can be identified. This nucleus is roughly oval in frontal sections and extends for approximately 300  $\mu$ m rostrocaudally. Posteriorly, it merges imperceptibly with undifferentiated parts of the hypothalamic gray. Rostrally, the anterodorsal preoptic nucleus arches dorsally in front of the anterior commissure toward the ventromedial tip of the ventral part of the lateral septal nucleus (Swanson and Cowan, '79). The latter has been called the dorsal part of the "septo-hypothalamic nucleus" by Bleier et al. ('79, '82). Loo ('31) included the anterodorsal nucleus along with areas more caudally under the name "anterior preoptic nucleus." Rose ('35) described a similar region, which he called the "nucleus hypothalami anterior intermedius," and which corresponds in general to Young's ('36) "nucleus of the anterior commissure." The anterodorsal nucleus corresponds to the part of Bleier's septo-hypothalamic nucleus that was described as persisting at the ventral border of the anterior commissure in the medial preoptic area (Bleier et al., '79). The anterodorsal nucleus is regarded here as a separate region, because the compact, darkly staining neurons in the ventral lateral septum have a clearly different morphology from the larger, more irregularly shaped neurons in the anterodorsal nucleus. Furthermore, the cells of the anterodorsal nucleus never merge with those of the ventral lateral septum, but remain separated by a distinct cell-sparse zone in the rostral part of the medial preoptic area.

A triangular region of low cell density lies just lateral to the anterodorsal preoptic nucleus and contains the precommissural component of the stria terminalis that passes beneath the anterior commissure. This region was studied in detail by Raisman and Field ('73), who referred to it as the "strial part of the preoptic area." This is indicated in figure 39.2 by the abbreviation StA ("strial area"). Raisman and Field ('73) identified a hormonally sensitive sex difference in the synaptic contacts of this area. Between the strial area and the bed nucleus of the stria terminalis lies a well-defined nucleus composed of at least two distinct parts. A rostral spherical cell group, called the "round nucleus" by Raisman and Field ('73), is replaced caudally by a lens-shaped group of fusiform cells arranged in parallel. These two cell groups are here referred to collectively as the *parastrial nucleus* (figure 39.1A, B). This nucleus is often considered part of the bed nucleus of the stria terminalis, which wraps around its lateral aspect. However, preliminary analysis of autoradiographic material (Simerly et al., unpublished observations) indicates that the parastrial nucleus may not receive an input from

the amygdala, and therefore should not be considered part of the bed nucleus. This finding is in agreement with the results of a degeneration study by Raisman and Field ('73).

In caudal parts of the medial preoptic area a small cluster of large, darkly staining neurons, the *posterodorsal preoptic nucleus* (figure 39.1D), can be differentiated from the surrounding gray. It lies near the caudal tip of the parastrial nucleus, just ventrolateral to the anterior magnocellular part of the paraventricular nucleus (Swanson and Kuypers, '80). The posterodorsal nucleus is not always easy to differentiate from the preoptic continuation of the bed nucleus of the stria terminalis (Gurdjian, '27), which wraps around its lateral aspect. However, the posterodorsal nucleus appears to be a separate cell group on the basis of several anatomical criteria. Its cells are more darkly stained than are those in adjacent parts of the bed nucleus, and are separated from it by a thin fiber capsule. The latter feature is particularly striking when the nucleus is viewed in horizontal and parasagittal sections. In addition, the posterodorsal nucleus contains many cells that selectively accumulate steroids (Schoonmaker, personal communication), and it appears to be sexually dimorphic, being over twice as large in the male as in the female rat (Simerly, unpublished observations). At least part of the parastrial and posterodorsal nuclei appears to correspond to Gurdjian's medial preoptic nucleus a (see also Raisman and Field, '73).

Finally, we have identified an *anteroventral* preoptic nucleus which is located just rostral to the MPN, being separated from it by a relatively cell-free zone (figure 39.1A). Medial to the anteroventral preoptic nucleus, just caudal to the vascular organ of the lamina terminalis, a darkly stained cluster of cells in the rostral part of the periventricular preoptic nucleus can be distinguished. It has been reported to be sexually dimorphic, being slightly larger and more densely cellular in the female (Bleier et al., '82). To maintain consistency with Gurdjian's nomenclature, we have called this cell group the *anteroventral periventricular nucleus* (figure 39.1A). This nucleus is often included in a region called the "nucleus preopticus, pars suprachiasmatica" (König and Klippel, '63; Pfaff and Kleiner, '73; Stumpf et al., '75). We restrict the use of the term *suprachiasmatic preoptic nucleus* (figure 39.1A) to a small cell group that lies between the anteroventral periventricular nucleus and the optic chiasm. In the nomenclature of Bleier et al. ('82), the suprachiasmatic preoptic nucleus and anteroventral periventricular nucleus together constitute the "medial preoptic nucleus." Gurdjian ('27), Loo ('31), Rose ('35), and Young ('36) did not distinguish either nucleus within the periventricular preoptic nucleus as a whole.



**Figure 39.4**

At left, thionin-stained 30- $\mu$ m-thick horizontal section through the MPN of a male rat to show subdivisions of the nucleus and the relationship of the MPN to surrounding structures. At right, outline drawing to show boundaries of cell groups illustrated in the photomicrograph at left. Scale = 200  $\mu$ m.

### The Medial Preoptic Nucleus

**Cytoarchitecture** Our results, based on correlations between cytoarchitecture and the distribution of serotoninimmunoreactive fibers, indicate that the MPN consists of at least three major subdivisions. Photomicrographs of four representative levels through the MPN are shown in figure 39.1. The nucleus is divided along most of its length into a cell-dense, darkly stained medial part, and a less cell-dense lateral part. At mid-rostrocaudal levels through the nucleus (figures 39.1C, 39.3), a small, extremely cell-dense cluster of cells embedded in the medial division can be identified. At both the rostral and caudal tips of the MPN, only the lateral part of the nucleus is present. This can best be appreciated in horizontal sections through all three subdivisions (figure 39.4).

The *medial part of the medial preoptic nucleus* (MPNm) contains small to medium-sized cells that tend to form irregularly shaped clusters. These cells are packed somewhat more closely than the cells of most other regions of the medial preoptic area (figure 39.3). The *central part of the medial preoptic nucleus* (MPNc) is a small, rounded cluster of intensely stained cells within the substance of the MPNm. It is composed almost entirely of small neurons that appear strikingly uniform in shape. Many of the cells near the periphery of the MPNc are arranged with their axes tangential to the thin fiber capsule that surrounds the cell cluster as a whole. The MPNc corresponds to the “sexually dimorphic nucleus of the preoptic area” described by Gorski et al. ('80). The *lateral part of the*

**Table 39.1**

Volumetric estimates of the MPN subdivisions<sup>1</sup> in two male and two female rats (in mm<sup>3</sup>)

	Female A	Female B	Male A	Male B
MPNm <sup>2</sup>	0.098	0.073	0.131	0.199
MPNI	0.145	0.140	0.172	0.169
MPNc	0.005	0.003	0.020	0.025
MPN	0.248	0.226	0.305	0.393

<sup>1</sup> MPNm, medial subdivision of the MPN; MPNI, lateral subdivision of the MPN; MPNc, central subdivision of the MPN.

<sup>2</sup> MPNm volume not including volume of MPNc.

*medial preoptic nucleus* (MPNI) is notably less cell-dense than the two other subdivisions, and its cells tend to be more variable in size and shape than are the cells in either the MPNc or the MPNm.

The volume of each part of the MPN is distinctly sexually dimorphic. Table 39.1 lists volumetric data from two representative male and two representative female rats. The volumes were calculated from areas obtained by tracing camera lucida drawings of the MPN with a planimeter [(area  $\div$  magnification<sup>2</sup>)  $\times$  tissue thickness = volume] without previous knowledge of the sex of the animals. The drawings were made by one investigator after cytoarchitectonic criteria were agreed upon by each investigator in the project. Boundaries were determined on the basis of cytoarchitectonic features in the Nissl-stained section, and on the distribution of serotonin-immunoreactive fibers in the adjacent section (see below). Although these values are only estimates, they consistently show a sexual dimorphism in the volume of each part of the MPN in these

animals. The MPNc is considerably larger in males, while the MPNm is only somewhat larger compared to the homologous structures in females. Although the MPNI appears to be slightly larger in the male, it occupies a larger proportion of the MPN in the female. The MPN as a whole appears to be slightly larger in the male. These results suggest that the MPN is a sexually dimorphic complex with at least three cytoarchitecturally distinct subdivisions. The cell-dense subdivisions appear to occupy a proportionally larger region of the nucleus in the male, while the cell-sparse lateral subdivision occupies the major part of the nucleus in the female. It will be important to confirm these results in a large series of paraffin-embedded brains, where tissue distortion is less and morphological details are clearer than in the frozen sections used here.

**The Distribution of Serotonin-Immunoreactive Fibers** Many serotonin-immunoreactive fibers appear to enter the medial preoptic area by passing medially from the medial forebrain bundle in the lateral preoptic area, as described in an autoradiographic study by Azmitia ('78), who found that fibers arising in the region of the median raphe nucleus ascend in dorsomedial parts of the medial forebrain bundle before turning medially to end in the medial preoptic area. Figure 39.5 shows the distribution of serotonin-immunoreactive fibers at four representative levels through the MPN of the male and female rat. These drawings are representative of the distribution seen in five animals of either sex. A dense bundle of serotonin-immunoreactive fibers can be seen to leave dorsomedial parts of the medial forebrain bundle and pass medially into the medial preoptic area, sending a large number of fibers between the MPN and the bed nucleus, into the strial area. A majority of these fibers presumably enter the stria terminalis and project to the amygdala (Conrad et al., '74). It should also be noted that the density of stained fibers is somewhat less in the adjacent parastrial nucleus. Most serotonin-immunoreactive fibers appear to enter the MPNI at posterior levels, although at least some fibers enter the nucleus all along its length, particularly dorsolaterally. Once inside the nucleus, the fibers ramify extensively throughout the MPNI, forming a dense plexus of varicose serotonin-immunoreactive fibers. The lateral border of this plexus defines the lateral border of the MPN. In contrast, the cell-dense MPNm contains few immunoreactive fibers, while the MPNc is virtually devoid of stained fibers. Thus, there appears to be an inverse relationship between cell density and the density of serotonin-immunoreactive fibers in the MPN.

The distribution of immunoreactive fibers in the MPN exhibits a gross sexual dimorphism, which correlates quite well with the sexual dimorphism observed in

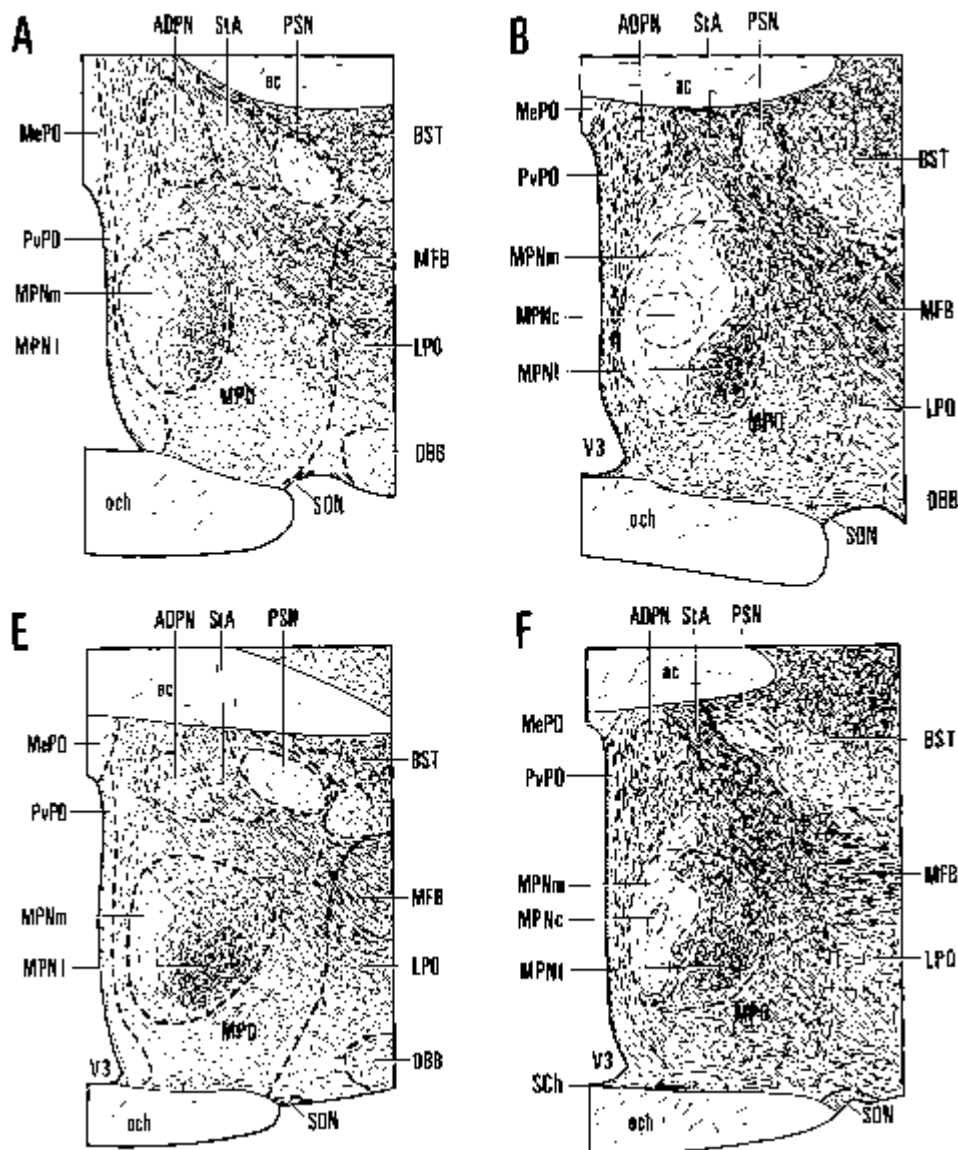
the size of the cytoarchitectonic subdivisions (figures 39.6, 39.7). In the male, a large area of low fiber density corresponds to its larger MPNm and MPNc, while in the female, a much smaller area of low fiber density corresponds to its smaller cell-dense subdivisions. Similarly, in the female a proportionately larger area of high fiber density, corresponding to its larger MPNI, is found, whereas in the male, a proportionately smaller area of high fiber density was found to correlate with its proportionately smaller MPNI.

The correlation between fiber density and the three cytoarchitectonic subdivisions of the MPN is illustrated graphically in the fiber density profiles shown in figure 39.8. In both the male and the female, the fiber density drops sharply near the medial border of the MPNm, is lowest in the MPNc, rises sharply near the lateral border of the MPNm, remains high in the MPNI, and declines near the lateral border of the MPN. The sexual dimorphism in the distribution of serotonin-immunoreactive fibers in the medial preoptic nucleus is equally apparent in the striking difference between the profiles of the male and female. The large area of low fiber density in the male contrasts with the smaller area of low fiber density found in the female. Similarly, a difference in the area of high fiber density is notable between the two sexes. The MPN of the female, with its proportionately larger MPNI, contains a correspondingly larger area of high fiber density. Similar findings were obtained in each of the animals used in this study; larger regions of low fiber density, corresponding to the MPNm and the MPNc, were found in the males, while larger regions of high fiber density, corresponding to the MPNI, were found in the females.

## Discussion

The results of the present study can be divided into two parts. First, we have tried to clarify the cytoarchitectonic organization of the medial preoptic area and at the same time introduce a consistent and simplified nomenclature, taking into account the previous literature. And second, we have shown that the medial preoptic nucleus is a sexually dimorphic complex that contains three major subdivisions, each of which has a characteristic distribution of serotonin-immunoreactive fibers. It is possible, therefore, to define sexually dimorphic cell groups within the MPN using two separate anatomical criteria. Taken together, these results should aid further studies of the morphology of the MPN, as well as provide a framework for functional investigations.

These results are in good agreement with those of Saavedra et al. ('74), who measured serotonin levels in punches from the medial and lateral preoptic areas of male Sprague-Dawley rats and found low levels of



**Figure 39.5**

A series of line drawings through the rostrocaudal extent of the MPN in the male (A–D) and female (E–H) rat to illustrate the distribution of serotonin-stained fibers in the MPN and adjacent regions at comparable levels in the two sexes.

serotonin in the former and relatively high serotonin levels in the latter. As expected, there was good correlation between our results and those of Steinbusch ('81; Steinbusch and Verhofstad, '84), who found a low density of serotonin-immunoreactive fibers in medial parts of the medial preoptic nucleus of Jacobowitz and Palkovits ('74), and in medial parts of the medial preoptic area in male rats. A medium density of stained fibers was reported (Steinbusch, '81) in the lateral part of the medial preoptic nucleus of Jacobowitz and Palkovits ('74), which appears to correspond, in general, to the MPNI of the present account. At central levels through the MPN, Steinbusch ('81) also indicated a small region of high fiber density that may correspond to the

plexus of immunoreactive fibers filling the MPNI, where fiber density is maximum (figure 39.5C).

Our results are somewhat different, however, from the histofluorescence findings of Fuxe ('65), and the autoradiographic results of Chan-Palay ('77) and Descarries and Beaudet ('78). Fuxe reported "scattered, very fine" serotonin terminals in the medial preoptic area and no terminals in the lateral preoptic area, while Chan-Palay ('77) found low densities of labeled fibers in both regions. In the autoradiographic study of Descarries and Beaudet ('78), moderate to high densities of labeled fibers were described in the medial preoptic area, and no labeled fibers were found in the lateral preoptic area, although in a more recent study (Parent

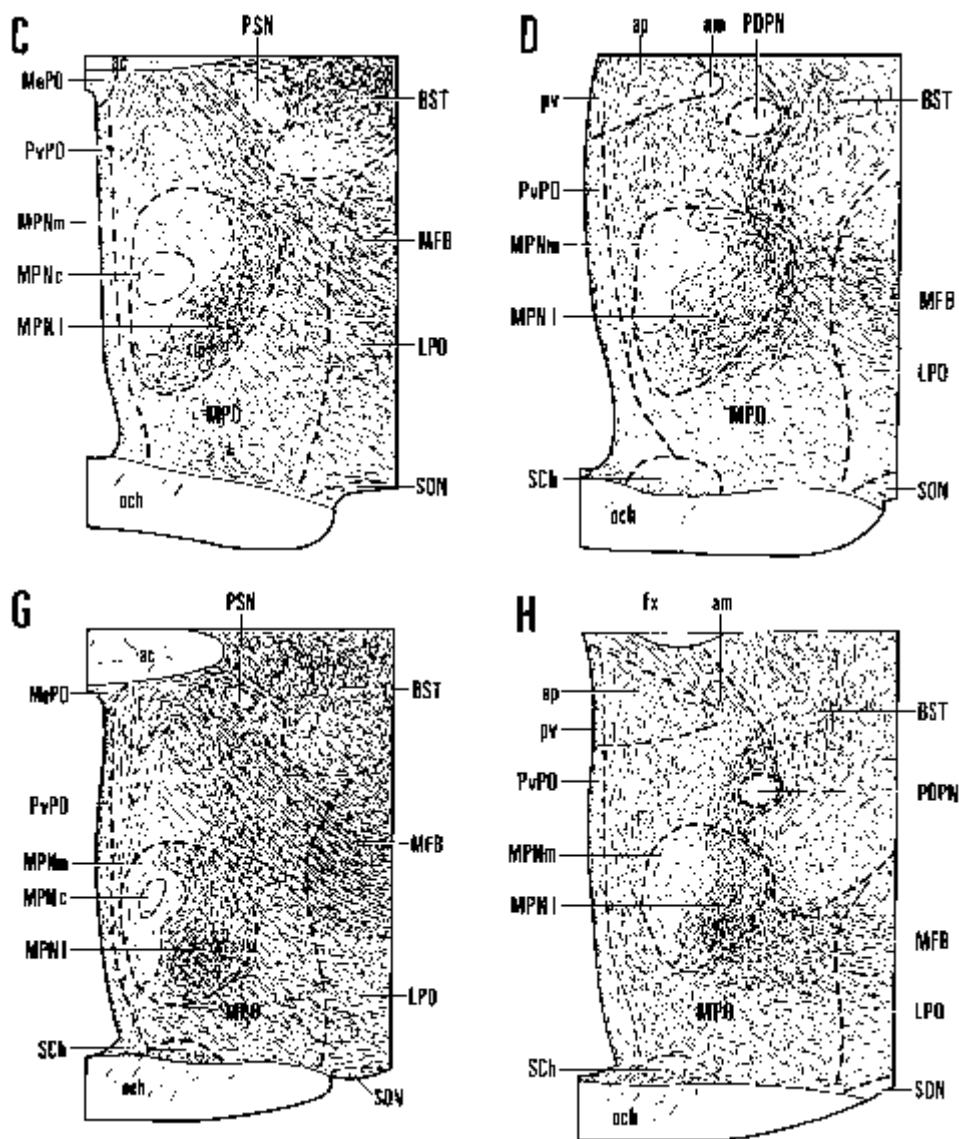
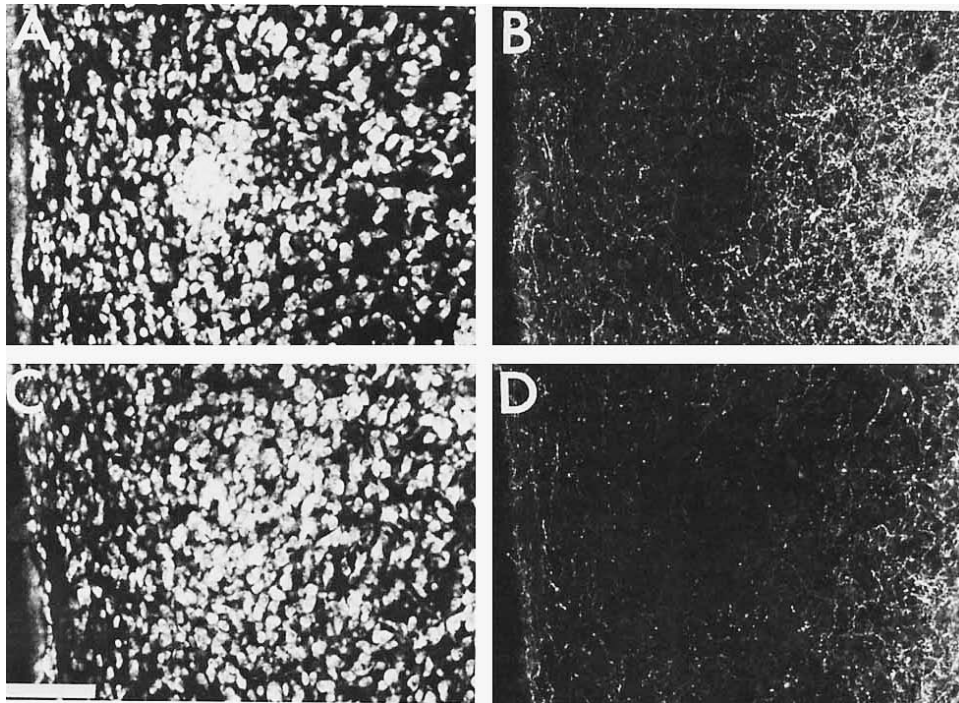


Figure 39.5  
(continued)

et al., '81), autoradiographic results were reported that appeared to be generally in agreement with those of Steinbusch ('81). These discrepancies may be accounted for by the relative insensitivity of the formaldehyde-induced fluorescence and autoradiographic methods compared with the more sensitive and specific immunohistochemical method of Steinbusch ('81). Even when the histofluorescence procedure is accompanied by pharmacological manipulations aimed at enhancing the specific fluorescence of serotonin, it is relatively insensitive for serotonin fibers, while the autoradiographic method may label other idolaminergic, and perhaps even catecholaminergic cells and fibers as well. Steinbusch ('78) has demonstrated that his serotonin antiserum crossreacts less than 2% with 5-

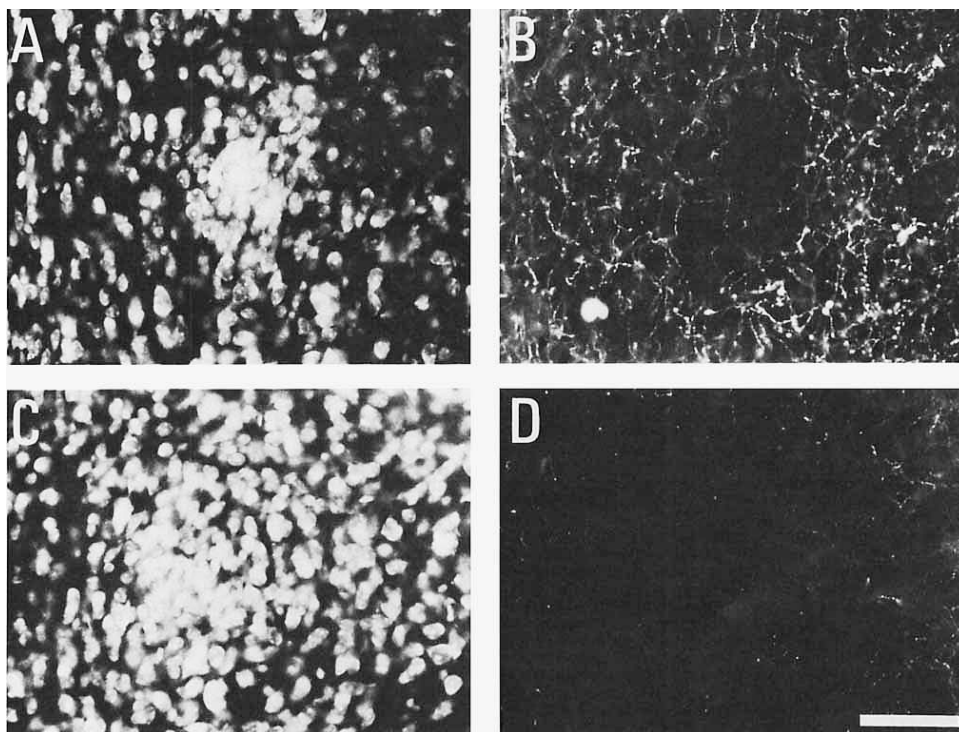
methoxytryptamine and dopamine, and less than 1% with noradrenaline, under the conditions used for immunofluorescence. Thus, it is unlikely that the staining observed in this study is due to nonspecific cross-reactivity with one of these other amines.

The cells that give rise to the sexually dimorphic serotonin-stained inputs to the MPN have not yet been identified with certainty. Berk and Finkelstein ('81) found retrogradely labeled cells in both the dorsal and median raphe nuclei following large injections of HRP centered in the medial preoptic area, and Conrad et al. ('74) and Moore et al. ('78) demonstrated efferents from both raphe nuclei to the medial preoptic area autoradiographically. By injecting  $^3\text{H}$ -proline into either the dorsal or the median raphe, and subsequently



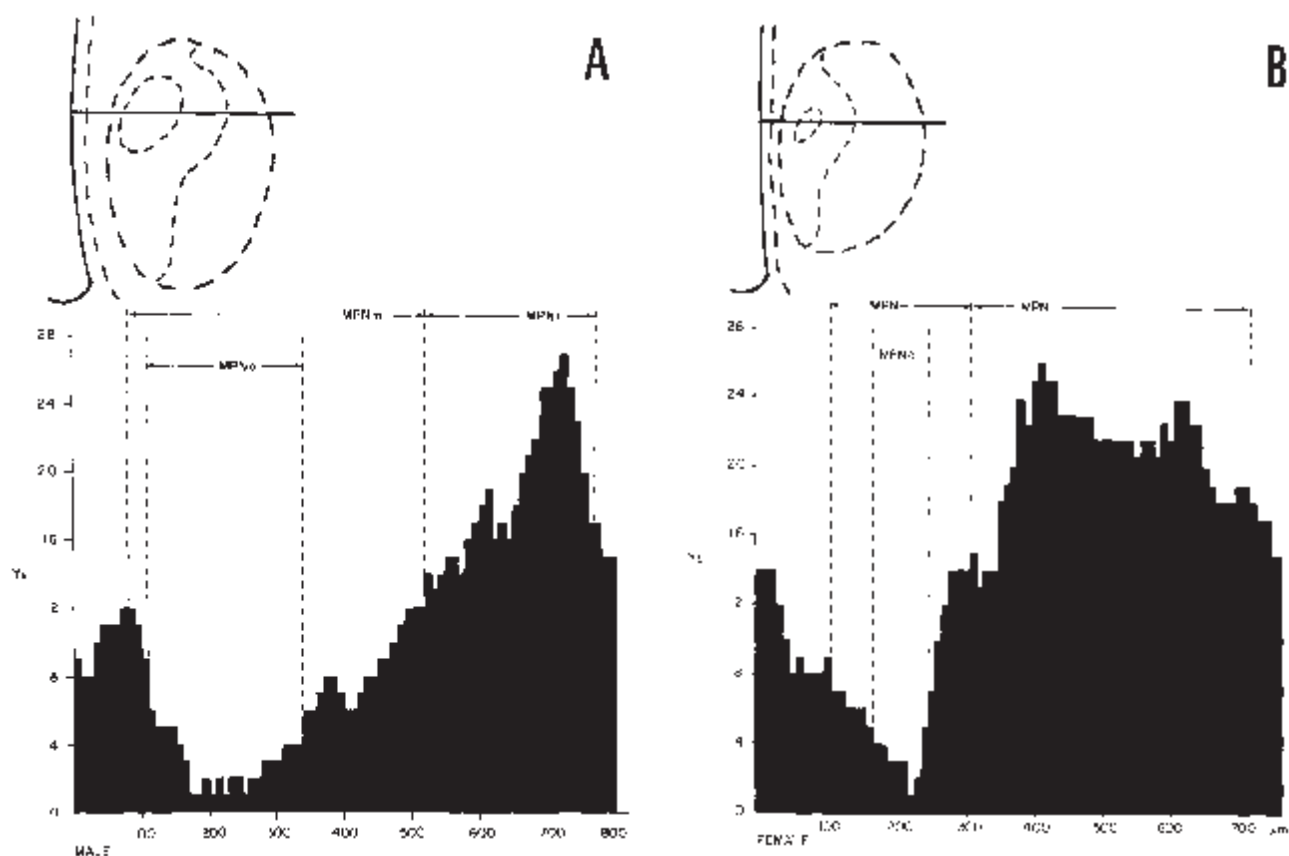
**Figure 39.6**

*A, B.* Two low-power fluorescence photomicrographs of the same field, taken with two different filter systems, to show the internal cytoarchitectonic details of the MPN (*A*) and the distribution of serotonin-stained fibers (*B*) in the female rat. *C, D.* Two fluorescence photomicrographs taken at the same magnification and at a comparable level to that in *A* and *B*, to show the cytoarchitectonic details of the MPN (*C*) and the distribution of serotonin-stained fibers (*B*) in the male rat. Scale = 125  $\mu$ m.



**Figure 39.7**

Four high-power fluorescence photomicrographs taken with two different filter systems that compare the cytoarchitecture of the MPNc (*A*) and the distribution of serotonin-stained fibers (*B*) in the female rat with that found in the male rat (*C* and *D*) at a comparable level through the MPN. Scale = 85  $\mu$ m.



**Figure 39.8**

Representative serotonin-stained fiber density profiles for male (A) and female (B) rats. The bold horizontal lines on the drawings above the histograms indicate the areas from which the traverses were made. The "bin width" used for the traverses was 10  $\mu\text{m}$ . The value plotted along the ordinate for each bin  $Y_x$  was obtained by calculating a four-bin running sum. The vertical dashed lines indicate the position and diameter of the MPN subdivisions along the traverses.

making unilateral injections of the neurotoxin 5,7-dihydroxytryptamine into the medial forebrain bundle, Azmitia and Segal ('78) deduced that the median raphe is the chief source of serotonergic afferents to the preoptic region. The neurotoxin injections virtually eliminated labeled fibers from the median raphe that ascend in the medial forebrain bundle through the lateral preoptic area to the medial preoptic area. Van de Kar and Lorens ('79) also found that lesions of the median, but not dorsal, raphe significantly reduced serotonin levels in the medial preoptic area. It is interesting that lesions of the median raphe did not completely eliminate serotonin in the medial preoptic area, indicating that it may receive a significant input from some other serotonergic cell group. Due to methodological limitations, and their relatively broad scope, it is difficult to extract information from these studies about serotonergic projections to the MPN itself. Whether serotonergic neurons in the median raphe contribute to the plexus that fills the MPNI, or whether there is a sex difference in the number or distribution of serotonergic perikarya that project to the MPN, re-

mains to be determined. The dense plexus of serotonin-stained fibers in the MPNI suggests that neurons in this region receive a serotonergic input. While it is clear that the MPNm and the MPNc receive few axosomatic serotonergic inputs, these cells may be influenced by the serotonergic system either by connections with interneurons in the MPNI, which does receive serotonergic afferents, or by contacts between the serotonin fibers and the dendrites of MPNm and MPNc cells that may extend into the MPNI (Greenough et al., '77).

The striking specificity of the sexually dimorphic, presumably serotonergic input, to the MPN is of particular interest since both the MPN and serotonin have been implicated in the control of male sexual behavior (Larsson, '79; Crowley and Zelman, '81). Although the role of the MPN in the control of female sexual behavior is unclear (Nance et al., '77; Clemens and Gladue, '79), there is good agreement in the literature that the MPN is essential for the display of normal male copulatory behavior (Heimer and Larsson, '66; Christenson et al., '77; Hansen et al., '82). In addition, when parasagittal knife cuts are made lateral to the

MPN, male sex behavior is severely interfered with (Szechtman et al., '78). Although such cuts would sever the serotonergic pathway from the medial forebrain bundle to the MPN (figure 39.5), it should be noted that these cuts could also affect other inputs, as well as a major projection pathway from the medial preoptic area (Swanson, '76). Finally, electrical stimulation of the medial preoptic area leads to decreased lordotic-responsiveness in female rats (Napoli et al., '72), while electrical stimulation in male rats results in increased male sexual behavior (Van Dis and Larsson, '70; Malsbury, '71; Merari and Ginton, '75). Effective sites for eliciting stimulatory effects on male sex behavior appear to be centered in medial parts of the MPN (Merari and Ginton, '75).

Serotonin has been reported to exert an inhibitory influence on both male and female sexual behavior (see Crowley and Zelman, '81, for review). Thus, injections of serotonin into the medial preoptic area of female rats resulted in significant decreases in lordosis behavior (Foreman and Moss, '77), and implants of serotonin antagonists into the same area increased lordotic-responsiveness in female rats (Ward et al., '75). In contrast, Soderston et al. ('78) did not observe effects on lordosis behavior following intracerebral treatment with the serotonin neurotoxin p-chloroamphetamine or with the neurotoxin 5,7-dihydroxytryptamine, either before or after treatment with estrogen and progesterone. Several investigators have reported that treatment of male rats with the tryptophan hydroxylase inhibitor p-chlorophenylalanine (Malmnäs and Meyerson, '71; Gessa and Tagliamonte, '75; Söderston et al., '76), or 5,7-dihydroxytryptamine (Söderston et al., '78) facilitates male copulatory behavior, suggesting that serotonin exerts an inhibitory influence on male sex behavior as well.

The serotonergic system has also been implicated in the control of another sexually dimorphic function, gonadotrophin release. Although there is general agreement that serotonin is involved in the control of luteinizing hormone release, its precise role is not well understood. Serotonin has been reported to both inhibit (Carrer and Taleisnik, '70; Barofsky, '75; Arendash and Gallo, '78; Waloch et al., '81) and facilitate (Héry et al., '78; Meyer, '78; Coen and MacKinnon, '79; Wilson and Endersby, '79) luteinizing hormone release. Functional studies directed at determining the role of the medial preoptic area in luteinizing hormone release are no less equivocal. Electrical stimulation within the medial preoptic area increases luteinizing hormone secretion (Kalra et al., '71), although lesions of the medial preoptic area that do not damage important parts of the gonadotrophin-releasing hormone pathway that arise more rostrally (King et al., '82) do not appear to significantly affect luteinizing hor-

mon secretion patterns (Nance et al., '77; Ryan and Frankel, '78; Wiegand and Terasawa, '82). The results of these functional studies, together with the anatomical findings presented here, suggest that the MPN may be at least one of the sites in which serotonin exerts an inhibitory influence on sex behavior, and perhaps more indirectly, influences the control of luteinizing hormone release. It should be remembered, however, that both the medial preoptic area and serotonin have been implicated in a great many functions, and that the MPN may serve as a nodal point in the neural circuitry underlying any, or all, of these functions.

It is interesting to note that the serotonergic innervation of the MPN is sexually differentiated, although present in both the male and female. It has been argued that since it is possible to "unmask" heterotypical behavior in both the male and female rat through hormonal manipulation, the neural substrate for both male and female sexual behaviors must be present in each sex (Gorski et al., '79). The finding that serotonin inhibits male sexual behavior in both male and female castrated rats (Söderston et al., '78) is consistent with this suggestion. Thus, the basis for observed sexually dimorphic behaviors may reside in the balance of sexually differentiated neural elements, which are present in each sex in differential amounts, and which exert their influence on the expression of the behavior in response to a specific hormonal state or neural input. The sexually dimorphic serotonergic innervation of the MPN may prove to be just such a neural substrate.

A number of other morphological sexual dimorphisms have been reported (see Arnold and Gorski, '83 for a review) and may be equally important in the regulation of sexually dimorphic neuroendocrine and behavioral functions. However, the specific functional roles of these sex differences have proven difficult to determine. It is known that each morphological sexual dimorphism is located in an area that contains steroid-concentrating cells (Pfaff and Keiner, '73; Sar and Stumpf, '75; and Stumpf et al., '75). This fact may be related to the role of steroids in development (Gorski, '83) or to the possibility that cells in these areas mediate endocrine influences on sexually differentiated brain function (McEwen and Pfaff, '73).

It has been suggested that serotonin is involved in the control of neurogenesis (Dörner, '80; Hamon and Bourgoignie, '82). For example, it has been proposed that serotonin may act as a "differentiation-signal" regulating the time of neurogenesis in cell populations that receive a serotonergic innervation in the adult (Lauder et al., '80). Furthermore, neonatal treatment with p-chlorophenylalanine has been reported to increase sexual behavior in adult male rats (Hyppä et al., '72). Although the details of this interaction are as yet unknown, the possibility that the sexually

dimorphic serotonergic fiber plexus in the MPN may be causally related to the cytoarchitectonic sex difference reported earlier (Gorski et al., '78) is worth pursuing. Crowley et al. ('78) have described sex differences in catecholamine content in certain brain nuclei that are permanently modified by changes in perinatal androgen levels. Similarly, a morphological sex difference in the density of vasopressin-immunoreactive fibers in the lateral septum (DeVries et al., '81) has recently been shown to be dependent on neonatal androgen (DeVries et al., '83). Whether the sexual dimorphism in the distribution of serotonin-stained fibers reported here is similarly affected by gonadal steroids remains to be determined.

A great deal remains to be learned about the neural circuitry underlying sexually dimorphic behavior before the correlations presented here can be related to functional concepts. However, the apparent coupling of a sexually dimorphic medial preoptic complex with a biochemically specific sexually differentiated neural input may provide us with a useful model system for studying the functional anatomy of sexually dimorphic behaviors.

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## Introduction

Both the medial preoptic nucleus (MPN) and serotonin (5-HT) have been implicated in the control of sexually differentiated physiological mechanisms and reproductive behaviors. The results of lesion (1, 15), pharmacological and behavioral studies (2, 3) suggest that the MPN may be at least one of the sites at which 5-HT influences sex behavior, and perhaps more indirectly, may affect the control of gonadotropin release (12, 26).

Previously we reported that the MPN in the rat is a sexually dimorphic complex composed of at least three subdivisions, each containing a characteristic distribution of 5-HT-immunoreactive fibers (20). The cell-dense medial part of the nucleus (MPN<sub>m</sub>) contains a low density of 5-HT-stained fibers, while the cell-sparse lateral part of the MPN (MPN<sub>l</sub>) contains a relatively dense plexus of 5-HT-immunoreactive fibers. Embedded within the MPN<sub>m</sub> is a very cell-dense central part of the nucleus (MPN<sub>c</sub>) that is nearly free of 5-HT-stained fibers, and corresponds approximately to the sexually dimorphic nucleus of the preoptic area described by Gorski and his colleagues (10). It has been shown that the volume of the sexually dimorphic nucleus in female rats can be completely masculinized by perinatal administration of testosterone propionate (TP) (6). It is not known, however, if this treatment has a similar effect on the entire MPN complex, or if the sexually dimorphic distribution of 5-HT-immunoreactive fibers is determined, at least in part, by the perinatal steroid environment. Recently, an immunohistochemical method has been used to show that a morphological sex difference in the density of vasopressin-immunoreactive fibers in the lateral septum (5) is dependent on neonatal androgen (4).

The present study was undertaken to test the sensitivity of the 5-HT-stained fiber distribution to the organization and activational (11, 16) effects of perinatal androgen exposure. By treating animals perinatally with TP, we attempted to imitate as closely as possible at least the temporal aspects of the naturally occurring exposure of male fetuses to testicular testosterone,

which is thought to affect the sexual differentiation of behavior and physiology, as well as brain morphology (8, 11), although the exact mechanism of this process is unknown. The distribution of 5-HT-immunoreactive fibers in the MPN of adult female rats that were treated postnatally or perinatally with TP and gonadectomized as adults was compared with the fiber distribution found in untreated females and males that were also gonadectomized as adults. The results were then compared with those obtained in a previous study using intact animals. In addition, the 5-HT-stained fiber distribution was compared with the cytoarchitectonic features of the MPN in each animal to determine if both morphological features are affected by perinatal testosterone exposure, and further, whether they show a similar or a differential sensitivity to this androgen treatment.

## Materials and Methods

Regularly cycling female Sprague–Dawley rats were placed in a cage with males on the day of proestrus. The following day sperm was seen in the vaginal smear and this day was designated as day 1 of gestation. Five groups of animals were compared in this study.

### Group 1: Female Animals Treated Perinatally with Testosterone Propionate (TP)

Rat fetuses were exposed to TP prenatally by giving daily subcutaneous injections of 2 mg TP (in 0.1 ml sesame oil) to the pregnant dams beginning on day 16 of gestation and continuing through the day of birth. The pups of these animals were injected subcutaneously with 0.1 mg TP (in 0.05 ml sesame oil) each day beginning on the day of birth and continuing through the 10th postnatal day. At 60 days of age the animals were gonadectomized, at which time the sex of the animals was determined. Male animals treated with TP were eliminated from the litters. Control animals that were treated perinatally with oil vehicle alone during the same time period were utilized in the control groups described below (groups 3 and 4).

### Group 2: Female Animals Exposed to a Single Dose of TP Postnatally

Pups from untreated pregnant dams received one subcutaneous injection of TP (0.1 mg TP in 0.05 ml sesame oil) on the 5th postnatal day. Oil treated control animals from these litters were combined with the perinatally oil-treated control animals to form groups 3 and 4.

### Group 3: Oil-Treated Females

Female animals that received either perinatal or postnatal injections of oil vehicle alone were combined in this group since preliminary results indicated that there were no marked differences in 5-HT-staining in the MPN between animals receiving either of these two treatments. The control females in this group were gonadectomized at 60–65 days of age.

### Group 4: Oil-Treated Males

Male animals treated with oil perinatally or postnatally were combined in this group. At 60 days of age these animals were gonadectomized.

### Group 5: Intact Females

The intact normally cycling female rats in this group were shown to be in estrus on the day of sacrifice by vaginal smear.

Two to 3 months after gonadectomy, each group of animals was sacrificed and processed for immunohistochemistry with an antiserum directed toward 5-HT as described in detail elsewhere (18, 24). The anti-5-HT was a gift from Dr. H. W. M. Steinbusch who has previously published details about its preparation and specificity (21, 22). Following immunohistochemical processing, sections were mounted, counterstained with ethidium bromide (19), coverslipped, and examined with a Zeiss Universal fluorescence microscope that allows independent visualization of the 5-HT-immunoreactive fibers and the cytoarchitectonic details of the MPN in the same tissue section, as described previously (20). The distribution of 5-HT-stained fibers was mapped onto camera lucida tracings from the thionin-stained reference series. To quantify the effect of the androgen treatments on the distribution of 5-HT-immunoreactive fibers in the MPN, fiber counts were made at 10  $\mu\text{m}$  intervals (in 100  $\mu\text{m}^2$  squares) along a defined traverse across the nucleus at a magnification of  $\times 500$ . Fiber density profiles were constructed for each animal and compared.

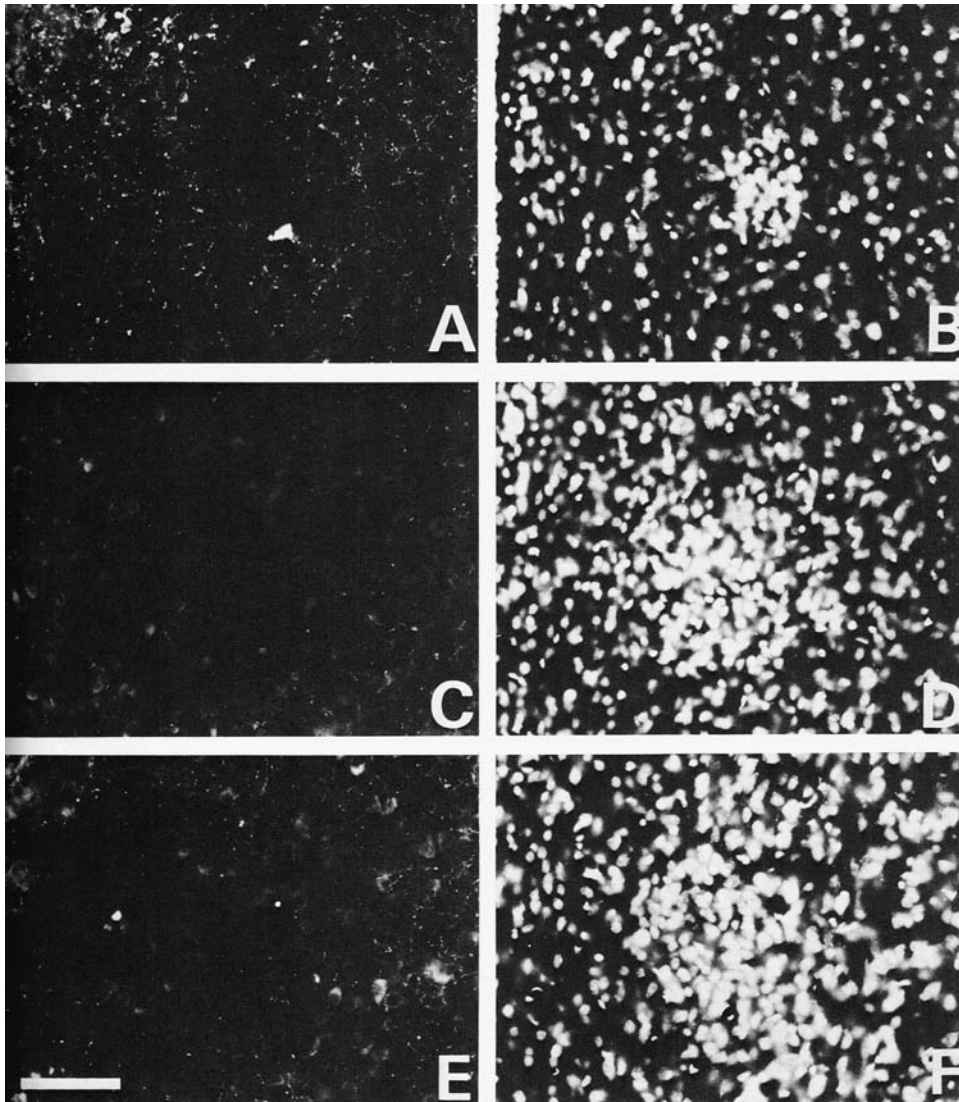
## Results

The distribution of 5-HT-immunoreactive fibers was found to be completely masculinized in each female rat that was treated perinatally with TP, while the mas-

culinization appeared to be substantial, though less complete, in each animal treated with TP postnatally. Both the control males and the perinatally androgen-treated females consistently showed a large area of low 5-HT-stained fiber density that corresponds to the  $\text{MPN}_m$  and  $\text{MPN}_c$  (figure 40.1C–F), while a proportionally smaller region of high 5-HT-stained fiber density was localized to the  $\text{MPN}_l$  in these animals. The 5-HT-stained fiber distribution pattern in both TP treatment groups contrasted greatly with that found in oil-treated females. In the control females, a notably smaller region of low 5-HT-stained fiber density and a proportionally larger region of high fiber density was found in the MPN. The distribution pattern is similar to that found in intact females (20) and is consistent with the presence of a smaller  $\text{MPN}_c$  and  $\text{MPN}_m$  (figure 40.1A, B) and a proportionally larger  $\text{MPN}_l$ . Thus, the perinatal androgen treatment appears to have completely sex-reversed the distribution of 5-HT-immunoreactive fibers in the MPN. This conclusion is supported by the fiber density profiles shown in figure 40.2. The smaller region of low fiber density and larger region of high fiber density of the control females (figure 40.2B) contrasts with the larger regions of low fiber density and smaller regions of high fiber density found in the control males (figure 40.2A) and in perinatally TP-treated females (figure 40.2C). The postnatally TP-treated females (figure 40.2D) showed a distribution pattern that was intermediate between that of normal males and females.

A similar masculinization of the relative sizes of the MPN subdivisions was seen in the TP-exposed animals. At comparable levels through the nucleus, the diameters of the MPN subdivisions in the perinatally TP-treated females were virtually identical to those measured in control males (figure 40.2A, C). In the postnatally TP-treated animals, the diameters of the subdivisions were notably larger than those of the oil-treated females; however, they remained smaller than those of either the control males or intact males (figure 40.2B, D). Although it seems clear that a significant masculinization of the volumes of the MPN subdivisions occurred in both groups of TP-treated animals, this must be verified quantitatively in a large series of paraffin-embedded brains where tissue distortion is less and the cytoarchitectonic details more distinct than in the frozen sections used in this study.

Adult gonadectomy did not appear to affect significantly either the distribution of 5-HT-stained fibers or the relative sizes of the subdivisions of the MPN. No major differences were found when the results obtained from the untreated, gonadectomized animals in this study were compared with those found using intact animals in a previous study (20).



**Figure 40.1**

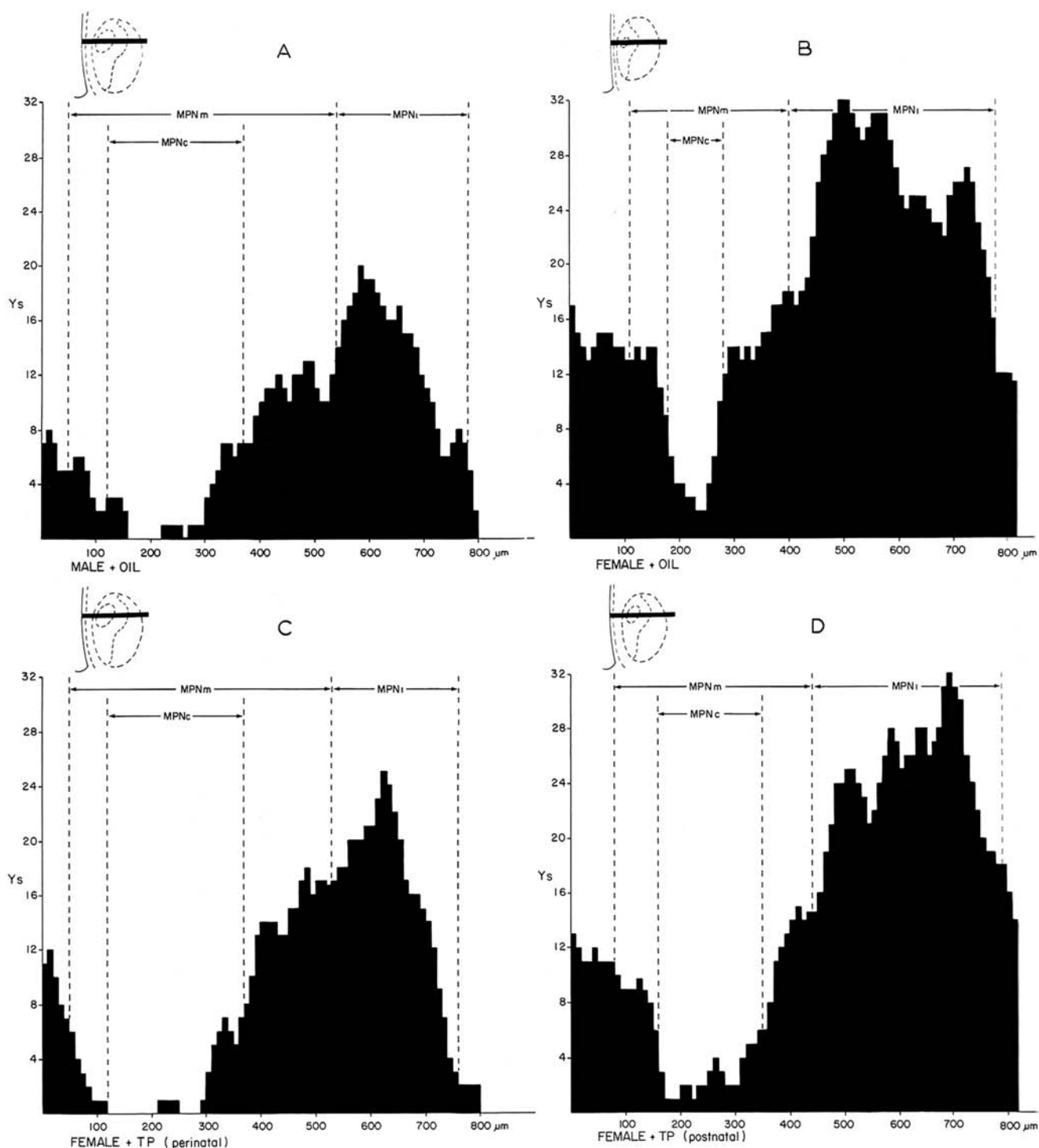
*A, C, E:* fluorescence photomicrographs that show the distribution of 5-HT-stained fibers in the MPN of an oil-treated female (*A*), an oil-treated male (*C*) and a perinatally TP-treated female (*E*) rat. *B, D, F:* fluorescence photomicrographs of the same fields shown in *A, C* and *E*, respectively, taken with a second filter system, to show the cytoarchitecture of the MPN<sub>c</sub> after counterstaining with ethidium bromide. Scale bar, 110  $\mu$ m.

As in intact animals, all of the animals in this study showed a close correlation between the distribution of 5-HT-stained fibers and the size of each MPN subdivision. In all animals the MPN<sub>m</sub> contained a low density of 5-HT-stained fibers in contrast to the relatively high fiber density found in the MPN<sub>l</sub>. In each case, the MPN<sub>c</sub> was virtually devoid of 5-HT-stained fibers. Thus, the apparent coupling of the distribution of 5-HT-immunoreactive fibers with the sexually dimorphic cytoarchitectonic subdivisions of the MPN was preserved in the TP-exposed females. Further, the perinatal androgen treatment masculinized both sexual dimorphisms to a comparable extent in what appears to be a complete sex reversal of both morphological

features. This finding is summarized in the drawings in figure 40.3, which show the masculinizing effects of the perinatal TP exposure on both the MPN subdivisions and on the distribution of 5-HT-immunoreactive fibers within the nucleus.

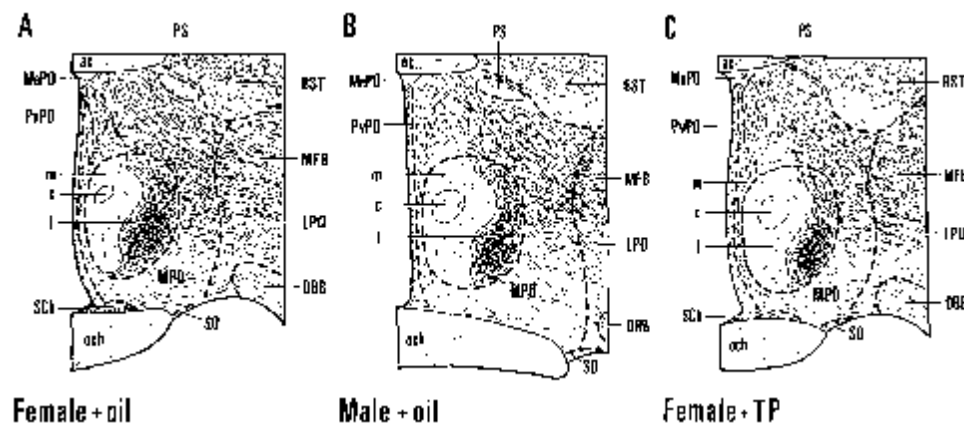
#### Discussion

These results suggest that the sexually dimorphic distribution of 5-HT-immunoreactive fibers in the MPN can be completely determined by the perinatal gonadal steroid environment. Although other factors may be involved in the natural sexual differentiation of this morphological feature, it is clear that large doses of



**Figure 40.2**

Representative fiber density profiles for oil-treated male (A), oil-treated female (B), perinatally (C) and postnatally TP-treated (D) female rats. The bold horizontal lines on the drawings above the histograms indicate the position of each traverse. The 'bin width' used for the traverses was 10  $\mu\text{m}$ . The value plotted along the ordinate for each bin, Ys, was obtained by calculating a 4-bin running sum. The vertical dashed lines indicate the position and the diameter of the MPN subdivisions along the traverse.

**Figure 40.3**

Line drawings of the medial preoptic area at the level of the MPN<sub>c</sub> to illustrate the distribution of 5-HT-stained fibers in the MPN and adjacent regions in oil-treated female (A), oil-treated male (B), and perinatally TP-treated female (C) rats. m, medial part of the MPN; c, central part of the MPN; l, lateral part of the MPN. For key to other abbreviations see ref. 20.

androgen administered perinatally can affect a complete masculinization of the 5-HT-stained fiber distribution in the MPN such that the distribution pattern is both qualitatively and quantitatively indistinguishable from that of normal males. Since postnatal treatment with TP resulted in a marked though incomplete masculinization of the 5-HT-stained fiber distribution, it would appear that, although sexual differentiation of the 5-HT-stained fiber plexus in the MPN largely occurs postnatally, the critical period may begin in the late prenatal period. However, whether postnatal TP treatment before day 5 can also effect a complete masculinization of the MPN remains to be determined.

In large tissue samples that included the forebrain and midbrain, higher levels of 5-HT, which appear to be sensitive to steroid manipulation, have been reported in the female as compared to the male rat (13). Recently, however, Watts and Stanley (25) were unable to find a significant sex difference in the 5-HT content of hypothalamic tissue samples. Although this appears to indicate that the steroid-sensitive sex difference in 5-HT content reported earlier (13) is extrahypothalamic, it is nevertheless possible that a sex difference in the 5-HT content of the MPN may be obscured in tissue samples that include the entire hypothalamus. Furthermore, the sex difference that we reported (20) is not based on 5-HT content, but rather on the distribution of 5-HT-stained fibers within the MPN, and may therefore be refractory to measurement in all but the most localized biochemical studies.

In addition to the masculinization of the 5-HT-stained fiber distribution, a concomitant masculinization of the cytoarchitecture of all 3 parts of the MPN was observed in the androgen-exposed animal. The dramatic increase in the size of the MPN<sub>c</sub> of TP-

treated females confirms the findings of Döhler et al. (6). These authors have recently reported that perinatal treatment of female rats with diethylstilbestrol also effects a complete masculinization of the sexually dimorphic nucleus of the preoptic area which suggests that aromatization of testosterone to estrogen may underlie the effects of gonadal steroids on this nucleus (7, 11).

Gonadectomy of the adult alone did not significantly affect either the distribution of 5-HT-stained fibers, or the cytoarchitecture of the MPN. This would seem to indicate that the observed masculinization is not due to activational influences of adult steroids on either the MPN or 5-HT neurons, but rather is the result of permanent, organizational effects exerted on the MPN by the perinatal steroid environment. The mechanism through which the steroids accomplish this masculinization is unknown. Both the MPN and the raphe nuclei contain steroid-accumulating cells (17, 23), but whether the steroids directly affect 5-HT neurons, or influence the sexual differentiation of this neural input at the level of postsynaptic neurons in the MPN, remains to be determined.

An interesting finding was the preservation of the apparent coupling of the 5-HT-fiber distribution with the sexually dimorphic MPN complex since the TP treatments masculinized both morphological features to a comparable extent. It has been proposed that 5-HT may act as a "differentiation signal" in cell populations that receive serotonergic inputs (14). Thus, it is conceivable that the 5-HT-fiber distribution and the sexual dimorphisms in the MPN complex may be causally related, and that the developmental interaction between the 5-HT neural system and this nucleus may be sensitive to changes in the perinatal steroid environment.

Hormonal manipulations can affect the expression of heterotypical behavior in both the male and female rat. This is consistent with the suggestion that the neural substrate mediating these functions must be present in both sexes (9), and that the basis for sexually dimorphic behaviors may reside in the relative differentiation of these substrates in a particular animal. Thus, the morphological effects of perinatal steroids on the sexually dimorphic distribution of serotonergic fibers in the MPN may relate to the imposition of a bias on a balanced system that influences the expression of a sexually dimorphic reproductive behavior or physiological function.

### Acknowledgements

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## Introduction

In 1971 Raisman and Field demonstrated that sex differences exist in the structure of the brain. To be precise, they found more spine synapses of non-strial origin in the medial preoptic area (MPOA) of female than of male rats. The years to follow yielded an ever growing list of morphological sex differences, e.g., in the size of specific brain regions, in dendritic and axonal branching patterns, and in the distribution of synapses. Most of these differences were shown to be dependent on the presence of androgens during a restricted period during development (for recent reviews see Gorski, 1984; Toran-Allerand, 1984). The functional meaning of some of these sex differences seems very clear, as for instance the presence or absence of the spinal motor nucleus of the bulbocavernosus in respectively male and female rats (Breedlove and Arnold, 1980): in male rats this nucleus contains motoneurons innervating the striated muscles of the penis, which are absent or vestigial in females. In most cases, however, it appears to be very difficult to elucidate the significance of a particular morphological sex difference. Several experimental procedures have been used in this respect, e.g. lesion studies, implantation of steroids, or attempts to find electrophysiological correlates (Dyer et al., 1976; Christensen and Gorski, 1978; Arendash and Gorski, 1983; Dyer, 1984). Up till now, such studies have made the topological correlation stronger of the site where sexually dimorphic functions are being regulated and the presence of morphological sex differences. For example, the MPOA—which shows sexual dimorphism with respect to synaptic patterns (Raisman and Field, 1971) and the size of the “sexually dimorphic nucleus of the preoptic area” (SDN-POA; Gorski et al., 1978)—has been further implicated in the regulation of gonadotropin release and male sexual behavior (e.g. Gorski, 1984). The functional implications of this structural dimorphism remain unclear.

An approach that might prove to be fruitful in obtaining a deeper insight into the nature and significance of the sex differences, is the study of the various neurotransmitter systems which form part of a given

morphological sex difference. Especially the use of immunocytochemistry might be of great help in this respect. Such a technique would not only facilitate the identification of the various components of a particular sexually dimorphic area, it can also be used to study the influence of various hormonal conditions on a specific neurotransmitter system. This chapter will first give a brief survey about various aspects of the study of sex differences in neurotransmitter systems. Then, as an example of the use of immunocytochemistry in studying sex differences, the vasopressin innervation of the brain will be discussed.

## Sex Differences in Neurotransmitter Systems

### Chemical Assays

The sex differences which are found in neuronal connectivity (for review see Toran-Allerand, 1984) suggest that such differences may also be expected with respect to the neurotransmitter content measured within sexually dimorphic areas. Knowledge about the neurotransmitter content, however, gives no information about the ongoing activity of the system being studied. The state of activity can be inferred from information about (i) the concentrations of the neurotransmitter and (ii) of its precursors and metabolites, (iii) the activities of synthesizing and degradative enzymes, and (iv) the receptor content. The presumed involvement of, e.g., monoaminergic and cholinergic systems in sexually dimorphic functions such as gonadotropin release and sexual behavior (Everitt et al., 1975; Hery et al., 1976; Clemens et al., 1981; Meyerson, 1984), suggests that in any case sex differences in the activity of these systems can be expected.

Sex differences have indeed been found in neurotransmitter content and enzyme activities of the cholinergic, noradrenergic, dopaminergic and serotonergic systems (Libertun et al., 1973; Gordon and Shellenberger, 1974; Vaccari, 1980; Luine and McEwen, 1983). Similar differences have been reported in the number of adrenergic, cholinergic and serotonergic receptors (Arimatsu et al., 1981; Avissar et al., 1981; Orensanz et al., 1982; Arimatsu, 1983; Fischette et al.,

1983). Sex differences in neurotransmitter systems were in fact known even before any structural sex difference in the brain had been demonstrated. To our knowledge, the earliest report is that of Kato, who demonstrated in 1960 that from the 68th day of life onward the serotonin content of the entire brain is higher in the female than in the male rat. This study has been repeated by various authors with more refined techniques, resulting in the description of sex differences in the content of serotonin, of its precursors and metabolites, and in the activity of the enzymes implicated in its metabolic pathways in a variety of rat brain areas (for review see Vaccari, 1980). Furthermore, elevated serotonin levels in the female brain have been reported to be present as early as the 12th postnatal day (Ladosky and Gaziri, 1970; Giulian et al., 1973). The early appearance of this sex difference is dependent on the neonatal presence of androgens in the male rat and, in turn, has been thought to be responsible for further sexual differentiation of the brain (Ladosky and Gaziri, 1970). This hypothesis would explain why it is that certain psychotropic drugs which interfere with the serotonergic activity of the brain, when given during pregnancy, can interfere with processes such as "defeminization" and "masculinization" of the brain (Jarzab and Döhler, 1984; a similar involvement in sexual differentiation has been claimed for the catecholaminergic system: Reznikov, 1978).

In addition to its use for tracing sex differences in neurotransmitter systems, chemical assays may give an indication about the influence of gonadal hormones on the activity of a certain neurotransmitter system. It therefore enables one to relate the neurotransmitter system in question to sexually dimorphic functions which vary consistently. For example, the administration of the dopamine agonist apomorphine reverses the decline in the display of male copulatory behavior which usually follows castration (Malmnas, 1977), whereas the neurotoxic destruction of the dopaminergic forebrain innervation in non-castrated male rats has detrimental effects on male sexual behavior (Caggiula et al., 1976). In addition, the administration of dopamine antagonists blocks copulatory behavior of castrated male rats, whether or not metabolites of testosterone—which normally stimulate this behavior (Baum and Starr, 1979)—had been simultaneously administered. These results suggest that under normal conditions testosterone facilitates male sexual behavior by stimulating the activity of dopaminergic neurons. This idea was corroborated by the fact that castration resulted in a decrease of dopamine concentrations in the septum and the nucleus accumbens septi, which could be reversed by administration of testosterone or of its metabolites estrogen and 5-dihydrotestosterone (Alderson and Baum, 1981; for similar studies see

Muth et al., 1980; Luine and Rhodes, 1983; Luine and McEwen, 1983).

Although this research on the dopaminergic innervation of the brain shows the value of chemical assays, one should bear in mind that most of the aforementioned methods are at present only applicable to a small number of monoaminergic, cholinergic and amino acid neurotransmitters, which probably forms only the top of the iceberg of the neurotransmitter population present in the brain. In addition, the chemical techniques do not allow a detailed morphological insight into the observed differences, and therefore are only of limited usefulness for clarifying the significance of a given morphological sex difference.

### Anatomical Studies

The study of the connectivity of a given sexually dimorphic brain structure may be of help for clarifying the function of this dimorphism. Examples of neuronal systems where such knowledge has indeed been indispensable in this respect are the previously mentioned spinal nucleus of the bulbocavernosus (Breedlove, 1984) and the vocal control areas in the bird brain (see e.g. DeVoogd, 1984). The anatomy of the latter system was originally studied by making lesions followed by a combination of behavioral and anatomical techniques (recording of song and detection of degenerating fibers, respectively; Nottebohm et al., 1976). These studies have demonstrated that this system was built of a series of interconnected forebrain and midbrain nuclei, which are connected to a medullar nucleus, which in turn contains motoneurons innervating the vocal organ: the syrinx. Some of these nuclei are much larger in male than in female birds, which is consistent with the fact that males sing, while females do not (Nottebohm and Arnold, 1976; DeVoogd, 1984). Although these examples demonstrate that unspecific methods such as anterograde and retrograde tracing techniques are of great value for determining the connections between specific brain areas, there are limitations to their usefulness. Difficulties arise, for instance, when one wishes to establish the source of the sexually dimorphic terminals in the MPOA, about which the only thing known is that they are of non-strial origin (Raisman and Field, 1971), or when one wants to describe the afferents and efferents of a relatively small area, such as the SDN-POA (Gorski et al., 1978). In such cases an approach which reveals the neurotransmitter systems present in the neuronal elements might be more appropriate since it might enable more refined anatomical studies.

Several methods have been developed for studying the anatomy of neurotransmitter systems (see e.g., Palay and Chan-Palay, 1982): (i) the histofluorescent demonstration of the indolamines and catecholamines, (ii) enzymes histochemical procedures for cholinergic

neurotransmitter pathways and (iii) immunocytochemistry, which is potentially the most versatile method, since it does not depend on detailed knowledge of either the chemical nature or the metabolizing enzymes for a given neurotransmitter. Moreover, it can be used for demonstrating an extremely wide variety of substances. The introduction of these methods has led to the discovery of many anatomical pathways in the brain which were not previously known (for review see e.g., Livett, 1978).

Recently these methods have been introduced in the study of sexually dimorphic vocal control nuclei in the zebra finch brain (Lewis et al., 1981; Ryan and Arnold, 1981; Ryan et al., 1981; DeVoogd, 1984), and a start has been made in exploring the various neurotransmitter systems which are present in the SDN-POA of the rat. Sladek et al. (1983) have demonstrated with histofluorescence that the catecholaminergic innervation of this structure appears denser in the female than in the male. Watson and Hoffman (1983) identified peptidergic components of the SDN-POA using immunocytochemical techniques. They found, for instance, that cholecystikinin-containing perikarya are present in the SDN-POA of both sexes, whereas substance P and neurotensin could be demonstrated only in males. Simerly et al. (1983), combining immunocytochemistry with a cytoarchitectonic study, demonstrated that the SDN-POA is virtually void of serotonergic innervation. In addition, they reported that the part of the MPOA immediately adjacent to the SDN-POA, contains a low density of serotonergic innervation (this part was larger in males than in females), while the remaining lateral part of the MPOA receives a relatively dense innervation (this latter part was smaller in males than in females). This study therefore shows that besides its use in clarifying the various components of known sexually dimorphic areas immunocytochemistry can reveal new morphological sex differences.

The aforementioned studies, which identify the various neurotransmitter components of a sexually dimorphic area, form only the onset to get a deeper insight in the anatomy and function of the sexually dimorphic areas. One way in which research on this topic might be pursued is exemplified by the study of the vasopressinergic innervation within the brain, to be discussed in the next section.

### **Sexually Dimorphic Vasopressin Pathways in the Brain**

#### **The Vasopressinergic Innervation of the Brain**

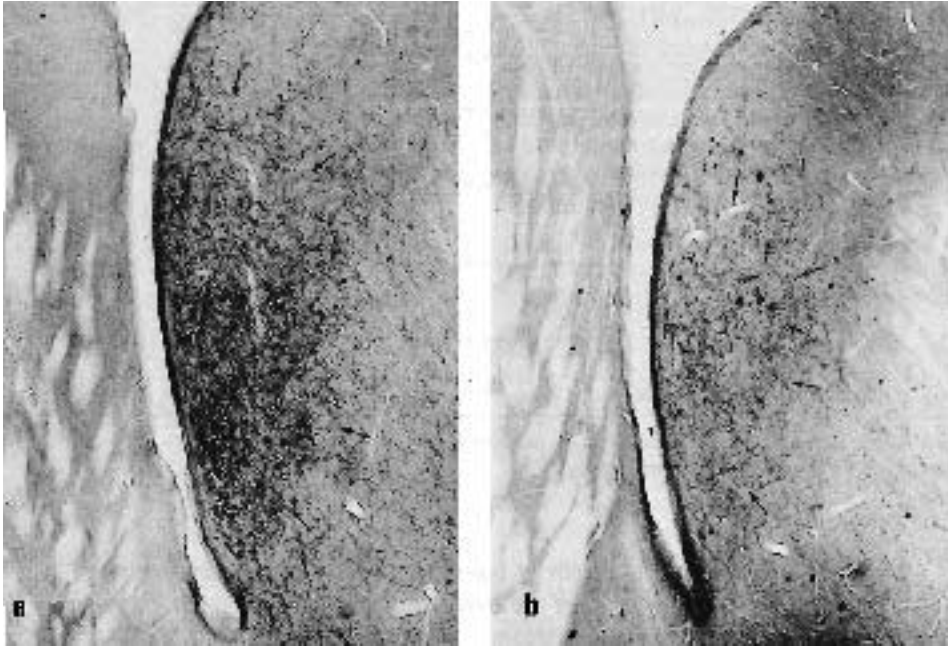
One decade ago vasopressin (VP) was only known to be present in the hypothalamoneurohypophyseal system. VP was believed to be synthesized exclusively in the hypothalamic paraventricular (PVN) and supraop-

tic nuclei (SON), from where fibers ran via the median eminence towards the posterior pituitary, where VP was released into the blood stream (Bargman and Scharrer, 1951). Immunocytochemical staining of sections of the rat brain for the presence of VP, however, revealed that also the parvocellular suprachiasmatic nucleus (SCN) contained VP (Swaab and Pool, 1975; Vandesande et al., 1975). Furthermore, VP fibers appeared not to be confined to the known neurosecretory pathways. In fact, they were found to innervate many areas throughout the brain, ranging from the olfactory bulb down to the spinal cord (Buijs, 1978; Sofroniew and Weindl, 1978). Limbic brain structures such as the lateral septum, lateral habenular nucleus and medial amygdaloid nucleus, appeared to be especially densely innervated by VP-containing fibers.

Several findings suggest that VP acts as a neurotransmitter in these areas. To begin with, VP fibers have been demonstrated to terminate synaptically on other neuronal elements in, e.g., the lateral septum and the nucleus of the solitary tract (Buijs and Swaab, 1979; Voorn and Buijs, 1983), from which areas VP can be released following physiological (Cooper et al., 1979) or depolarizing stimuli (Buijs and Van Heerikhuijze, 1982). When applied locally to these areas VP is able to change unit activity (e.g. Hywiler and Felix, 1980; Joëls and Urban, 1982; Mühlethaler et al., 1982) which effect can be blocked by vasopressin antagonists (Mühlethaler et al., 1982). VP-binding sites could in fact be demonstrated autoradiographically in several areas, notably in the lateral septum (Baskin et al., 1983; Van Leeuwen and Wolters, 1983). Since, finally, also VP-degrading mechanisms have been found in the brain (Burbach et al., 1983), VP has already met many of the established criteria for neurotransmitter identification (Barchas et al., 1978).

#### **Sex Differences and Influence of Gonadal Steroids**

As this peptide was suggested to be of possible importance for brain development (Boer et al., 1980), the ontogeny of the VP pathways in the brain was studied (Buijs et al., 1980; De Vries et al., 1981). In these studies no distinction was made at first between the two sexes. When studying the lateral septum and the lateral habenular nucleus, the first VP fibers were found on the 10th postnatal day. From the 12th postnatal day onwards large individual differences were found in the density of the innervation in these areas. In some animals the density remained very low while other animals developed a high VP fiber density. When, in a second experiment, the rats were separated according to their sex, this variation turned out to be due to a sex difference, the VP fiber density in the lateral septum and, to a lesser extent, in the lateral habenular nucleus being higher in male than in female rats



**Figure 41.1**

Transverse sections of the lateral septum of the rat stained immunocytochemically for the presence of vasopressin. Note that the density of the vasopressin fiber network (arrows) is higher in the male (a) than in the female rat (b). (Modified from De Vries et al., 1981.)

from the 12th postnatal day onwards (figure 41.1) (De Vries et al., 1981). To determine whether or not this sex difference was dependent on the neonatal presence of androgens, a series of castration and testosterone supplementation experiments was performed, after which the rats were examined on the 26th postnatal day (De Vries et al., 1983). At this age the normal difference between male and female rats is most pronounced. All of the male rats which had been castrated on the first day of life showed a VP fiber density in the lateral septum which was as low as in control females, i.e., hardly any fibers were found. In male rats castrated on the 7th postnatal day, a fiber density intermediate to that of control males and females was detected. When the rats were castrated on the 14th day of life, a fiber density was found which was equal to that in control males. These results suggested that in males the presence of androgens during the first 2 postnatal weeks caused the VP innervation to become denser than in females. The administration of testosterone propionate either to female or to neonatally castrated male rats, however, yielded results that pointed to another possibility. Although high doses stimulated the VP network in the lateral septum to become as dense as in normal males, it did not make any difference whether the testosterone was administered in the first, second or third week of life. This suggested that the plasticity of the system is great enough to permit an influence of testosterone even at later stages of life.

This has been recently confirmed in an experiment showing that in male rats which were castrated at 3 months of age, the fiber density in the lateral septum decreased gradually within a period of 15 weeks from very high to a level at which almost no fibers were found. When, at this point, the castrated rats received an implant of silastic tubing packed with testosterone, fiber density returned to the original level within 5 weeks (table 41.1). Ovariectomy of female rats appeared to have the same effect as castration in male rats (De Vries et al., 1984). Therefore, gonadal steroids seem to be of general importance for the maintenance as well as for the development of the VP fibers in the lateral septum.

#### **The Question of the Origin of the Sexually Dimorphic VP Innervation of the Lateral Septum**

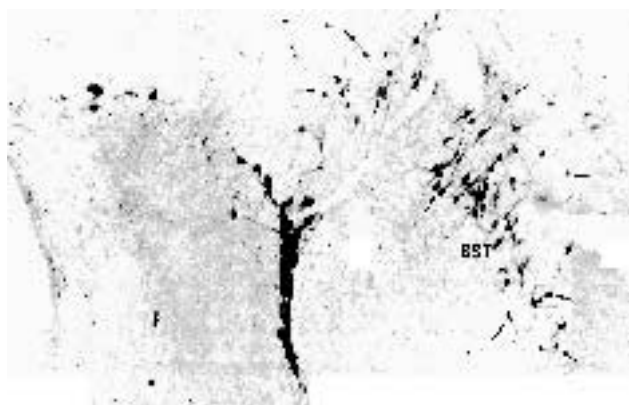
The source of all extrahypothalamic VP fibers was originally thought to be the PVN and SCN (Buijs, 1978; Sofroniew and Weindl, 1978). The SON seemed an unlikely candidate, since virtually all its efferents appear to run towards the neurohypophysis (Troiano and Siegel, 1975; Swanson and Sawchenko, 1983). However, lesioning the SCN or the PVN did not affect the VP fiber density in the lateral septum although these lesions eliminated the VP innervation of areas bordering the third ventricle and of areas in the hind brain (Hoorneman and Buijs, 1982; De Vries and Buijs, 1983). Retrograde studies confirmed these findings, because no retrogradely labeled cells were found

**Table 41.1**

VP fiber density in the lateral septum at several weeks after castration and subsequent testosterone supplementation in male rats

Weeks	Male Rats	Fiber Density						
1	Control	++++	++++	++++	++++	++++		
	Castrated	++++	++++	++++	++++	++++		
3	Control	++++	++++	++++	++++	++++		
	Castrated	++++	+++	+++	+++	+++		
8	Control	++++	++++	++++	++++	++++		
	Castrated	+++	++	++	++	++		
15	Control	++++	++++	++++	++++	++++		
	Castrated	+	+	+	+	+		
25	Control	++++	++++	++++	++++	++++		
	Castrated	+	+	+				
	Castrated plus testosterone	++++	++++	++++	++++	++++	+++	+++

Each entry in the table represents 1 rat: +, almost no fibers; ++, a low fiber density; +++, a high fiber density; +++++, a very high fiber density.

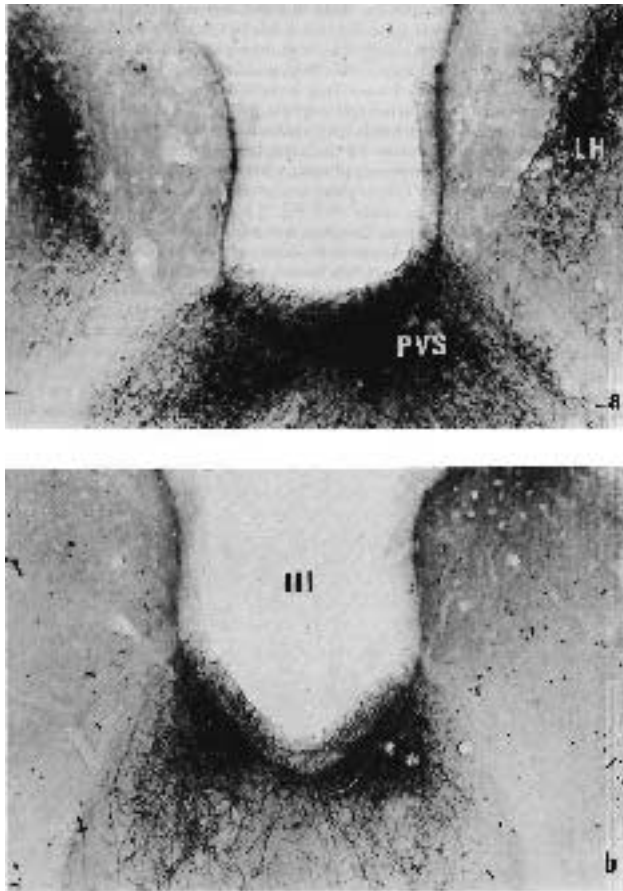
**Figure 41.2**

Transverse section of the region of the bed nucleus of the stria terminalis (BST) of the rat stained immunocytochemically for the presence of vasopressin (VP). Arrows indicate the VP cells which became visible after colchicine pretreatment. The magnocellular VP cells immediately adjacent to the fornix (F) are neurosecretory neurons which project to the neurohypophysis (Kelly and Swanson, 1980).

in the SCN, PVN and SON after placement of various tracers in the lateral septum (De Vries and Buijs, 1983). At that point all known VP-containing nuclei had been eliminated as plausible sites for the origin of the VP fibers in the lateral septum. Recently, however, VP-containing cells were found in still other places in the brains of rats which had been pretreated with colchicine (Caffe and Van Leeuwen, 1983; Van Leeuwen and Caffe, 1983). The site where most of the additional VP cells were detected was the bed nucleus of the stria terminalis (BST; figure 41.2). Some other groups were found in the medial amygdaloid nucleus, the locus coeruleus, and the dorsomedial hypothalamus. When the BST was lesioned a strong reduction in the density of the VP innervation of the lateral septum was indeed found. Other areas where the fiber density was affected, were the diagonal band of Broca, the lateral habenular nucleus, the medial amygdaloid nucleus, the periven-

tricular grey and the locus coeruleus (De Vries and Buijs, 1983).

Because these lesions might also have destroyed fibers of passage, ultimate proof for the VP projections of the BST can only be obtained after retrograde tracing in combination with the demonstration of VP in the labeled neuron (cf. Sawchenko and Swanson, 1982). Several findings, however, support the candidacy of the BST as source of the VP fibers in those areas where fibers were seen to disappear; (i) after injection of tracers in the lateral septum, retrogradely labeled cells were found in the same regions of the BST where VP cells were found in colchicine-treated rats (De Vries and Buijs, 1983; Van Leeuwen and Caffe, 1983), in addition (ii) anterograde tracing studies have shown that the BST sends projections to all areas where VP fibers were affected after BST lesions (Conrad and Pfaff, 1976), and (iii) the results of the previously mentioned castration and ovariectomy experiments provide an additional argument in favor of the BST: all areas to which the VP neurons of the BST presumably project, displayed a very strong regression in the VP fiber density following gonadectomy of adult male and female rats. After testosterone replacement therapy in the castrated males, VP fibers reappeared in these areas (figure 41.3) (De Vries et al., 1984). The stainability of VP neurons in the BST followed the same pattern, i.e., no cells were found in the long-term castrated rats, but they reappeared after testosterone replacement therapy (Van Leeuwen et al., in preparation). Interestingly, similar changes in the VP innervation were found in the medial amygdaloid nucleus, the ventral hippocampus and the ventral tegmental area. The origin of these VP fibers is unknown, although they might be derived from the most ventral part of the BST which was not affected in the previously mentioned lesion studies. An additional possibility is that VP cells in the medial amygdaloid nucleus form the source (Caffe and Van Leeuwen, 1983) since



**Figure 41.3**  
Transverse sections of the area around the third ventricle (III) stained immunocytochemically for the presence of vasopressin (VP), showing a dense VP fiber network in the lateral habenular nucleus (LH) of a control rat (a), in contrast to the absence of fiber staining in a rat at 15 weeks after castration (b). Note that this difference is not present in the periventricular nucleus (PVS).

these cells showed similar changes as the BST cells following hormonal manipulations (De Vries, in preparation). No changes following the hormonal manipulations were found in the VP projections from the SCN and PVN (for a schematic representation of the changes observed after gonadectomy, see figure 41.4). Taken together, these results demonstrate not only that the VP innervation of the brain can be subdivided as to origin, but also that these subdivisions respond differently to hormonal stimuli and, presumably participate in different ways in neurally regulated functions.

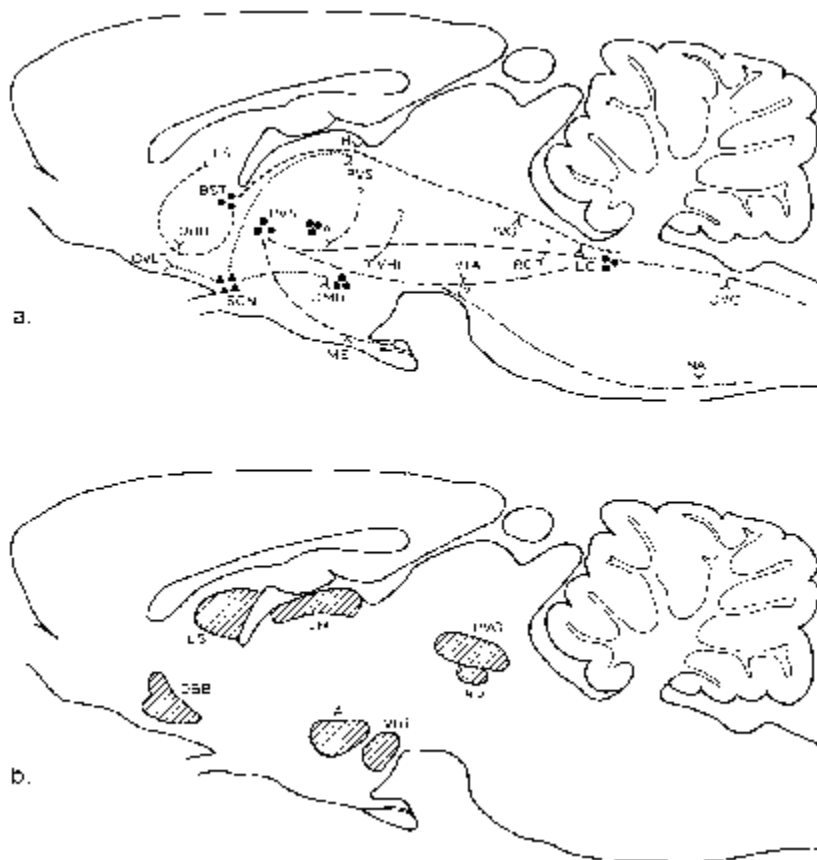
#### Questions on the Action of Gonadal Hormones on the VP Fiber Density

The site where gonadal hormones act so as to influence the VP projections of the BST is unknown. Gonadal steroid implantation studies (cf. Christensen and Gorski, 1978) might be used in order to shed light on this

problem. It might be that they act directly upon the BST, because many neurons in this area have been shown to concentrate gonadal steroids (Pfaff and Keiner, 1973; Stumpf and Sar, 1976). Such an action could be directed either to the VP neurons in this area, or at other neurons which in turn would influence the VP neurons. The latter situation might apply to those preoptic neurons which contain "luteinizing hormone-releasing hormone" (LHRH): although the fiber projections of these cells are affected by gonadectomy (Shivers et al., 1983a), a combination of autoradiography and immunocytochemistry has made it clear that almost no LHRH cells concentrate estradiol (Shivers et al., 1983b).

Besides the question of where gonadal hormones act so as to change VP projections, it is not clear in what way such changes should be explained. Should one see the disappearance and reappearance of fiber staining as an actual outgrowth and retraction of VP fibers or merely as changes in the state of filling of otherwise intact fibers following hormonal manipulations? The aforementioned effects of androgens on the developing VP innervation might be reasonably interpreted as a stimulation of nerve fiber outgrowth. Such a mechanism would then be in line with the observations of Toran-Allerand (1976, 1984) who has demonstrated that steroids promote neurite outgrowth in cultures of newborn mouse hypothalamus and in explants derived from the BST. That a similar process also takes place in adulthood, viz., a complete retraction of the VP fibers after gonadectomy and again an outgrowth after steroid replacement therapy, seems improbable. However, stimulation of dendritic growth under the influence of gonadal steroids has been demonstrated in the adult avian brain (DeVoogd and Nottebohm, 1981) while electron microscopic analysis has demonstrated that the mammalian brain too displays structural plasticity in adulthood, at least with respect to the number and nature of synaptic contacts (Dyer, 1984). As for the VP pathways, it could well be that the changes in fiber staining represent a combination of both possibilities, i.e., the axonal length and branching change in parallel with alterations in the state of filling of the fibers.

Another problem concerns the nature of the sex difference in the lateral septum. In the first place, also in this case one cannot say whether females really contain fewer VP fibers than do males, or simply that the VP content in many of the fibers is below the level of detection. Secondly, it is still not clear whether the sex difference is maintained in adulthood exclusively by differences in the hormonal milieu, or whether it reflects a sex difference in the brain that was established during development. An indication that the differences are not due solely to hormonal conditions in

**Figure 41.4**

(a) Scheme of the major vasopressin (VP) pathways in the brain with their most likely origin (see text). ———, pathways from the paraventricular nucleus (PVN); ..... pathways from the supraoptic nucleus (SCN); -----, pathways from the bed nucleus of the stria terminalis (BST). Squares, triangles, and dots indicate, respectively, VP cell groups in the PVN, SCN, and cell groups which were found only after colchicine treatment, e.g., in the BST. A question mark indicates that the source of the VP innervation in that particular area is still uncertain. (b) Scheme indicating the major (hatched) areas where VP fibers were seen to disappear after gonadectomy. Note that the fibers disappear from all areas to which the BST presumably projects and from the areas where the origin of the fibers is unknown. A, amygdala; DBB, diagonal band of Broca; DMH, dorsomedial nucleus of the hypothalamus; DVC, dorsal vagal complex; LC, locus coeruleus; LH, lateral habenula; LS, lateral septum; ME, median eminence; NA, nucleus ambiguus; OVLT, organum vasculosum lamina terminalis; PVG, periventricular grey; PVS, periventricular nucleus; RD, dorsal raphe nucleus; VHi, ventral hippocampus; VTA, ventral tegmental area. (Figure 41.4a from Buijs et al., 1983.)

adulthood is given by the fact that, whereas testosterone replacement therapy in adult castrated male rats restores the VP fiber density in the lateral septum to the original density, the same treatment can bring the fiber density in adult ovariectomized female rats only at the level characteristic for normal females (observations of Wouter Duetz, in our laboratory). If the sexual dimorphism is indeed inherent to a differentiated VP system, it might for instance be the consequence of sex differences in the ramifications of VP fibers or the number of VP cells in the BST. Interesting in this respect is, that about twice as many VP cells are found in male than in female rats after colchicine treatment (figure 41.4) (Van Leeuwen et al., in preparation). Again, the actual number of VP cells might be higher than that observed in the immunocytochemically stained sections, which possibility seems to be corroborated by the aforementioned disappearance of the cells after

gonadectomy and reappearance after testosterone replacement therapy. It is noteworthy, however, that a sex difference has been found in the BST of the guinea pig, comparable to that in the SDN-POA of the rat (Hines et al., 1983). This finding suggests that the BST of the rat might contain structural sex differences which could include VP cells as well.

#### Possible Functions of the VP Innervation of the Brain

The earlier mentioned classification one can make as to the origin of the VP innervation may give a lead as to what functions the various subdivisions might be involved in. The VP projections of the SCN follow closely all the projections of the SCN as established by anterograde tracing techniques (Berk and Finkelstein, 1981; Hoorneman and Buijs, 1982). Since the SCN plays a keyrole in the generation of circadian rhythms (Moore, 1978) and the estrous cycle (Brown-Grant and

Raisman, 1977) the VP projections of this nucleus might be involved in these rhythms as well.

VP projections of the PVN run predominantly to the medulla and the spinal cord where they innervate areas such as the nucleus of the solitary tract, which are involved in the regulation of autonomic functions (see, e.g., Palkovits and Zaborsky, 1977). These VP projections indeed may play a role in such functions, since central administration of VP influences blood pressure and heart rate, especially when injected into the hind brain (Bohus, 1980; Matsuguchi et al., 1982; Pittman et al., 1982). Since those areas project in turn to the PVN and SON, it is possible that the VP projections of the PVN influence, via a feedback loop the release of VP into the blood stream (see Buijs et al., 1983; Swanson and Sawchenko, 1980).

The observed sex differences and the changes under influence of hormonal manipulations in the remaining part of the VP fiber pathways (which for matters of convenience will be referred to as "the sexually dimorphic VP projections") suggest that these VP pathways are probably involved in functions which are strongly influenced by gonadal steroids. Such functions would, however, not necessarily have to play a part in reproductive processes like gonadotropin secretion or sexual behavior. A variety of neurally regulated functions such as feeding, learning, and aggressive behavior are sexually dimorphic or strongly influenced by gonadal steroids in adulthood (for reviews see Van de Poll et al., 1978; Goy and McEwen, 1980; Beatty, 1984). The sexually dimorphic VP projections could be involved in such an apparently non-reproductive but still sexually dimorphic function.

A good candidate for such a function is the regulation of VP release into the blood by the neurohypophysis: whereas VP secretion is rather constant in male rats, in females it follows a cyclic pattern, which is synchronized with the estrous cycle (Swaab and Jongkind, 1970; Skowsky et al., 1979). Furthermore, the administration of estrogen to gonadectomized rats stimulated VP release, while androgens inhibited it (Skowsky et al., 1979). There are indications that the sexually dimorphic VP projections to areas such as the septum and the amygdala are indirectly involved in the control of VP secretion by the neurohypophysis. Thus, both the septum and the amygdala send projections to the PVN and SON (Garris, 1979; Silverman et al., 1981; Tribollet and Dreifuss, 1981; Oldfield et al., 1983), and electrical stimulation of the former areas indeed results in a change in spike frequency of the neurosecretory cells of the PVN and SON (Negoro et al., 1973; Poulain et al., 1980).

The changes in peripheral VP levels which are induced by gonadal hormones might be caused by a direct action of steroids upon hypothalamic neuro-

secretory neurons. The VP-containing neurosecretory neurons of the PVN and SON of the mouse concentrate estrogens (Sar and Stumpf, 1980). In the rat, however, no estradiol-concentrating cells have been found in the SON, while in the PVN such cells were found only in regions containing predominantly oxytocin cells (Rhodes et al., 1981). The same regions contain only very few vasopressin cells most of which project to the medulla and spinal cord rather than to the neurohypophysis. This led the authors to conclude that in the rat gonadal steroids exert no direct action upon the neurosecretory neurons and therefore influence VP release via an indirect mechanism (Rhodes et al., 1981). If the sexually dimorphic VP projections indeed turn out to be implicated in the regulation of VP release, it will be of great interest to investigate whether or not the influence of gonadal steroids on VP release is mediated via this system.

### Vasopressin and Reproductive Functions

A clue to the function of the sexually dimorphic VP projections might be found in the dynamics of the observed changes in fiber density after gonadectomy (viz., a decrease over 15 weeks versus a restoration of the original fiber density within 4 weeks; results of Wouter Duetz in our laboratory). These changes are rather slow when compared with the changes in LHRH innervation in the midbrain central grey, which decreases drastically within a week following gonadectomy of male rats or after estrogen replacement in gonadectomized females (Shivers et al., 1983a). The time course of these last changes is consistent with the postulated involvement of LHRH fibers within the midbrain central grey in lordosis behavior (Riskind and Moss, 1979; Sakuma and Pfaff, 1980), since this behavior too disappears shortly after ovariectomy and reappears within a few days following estrogen administration (Harlan et al., 1984). By contrast, male sexual behavior decreases with a time course comparable to the changes in the density of the sexually dimorphic VP projections after castration (Davidson, 1966). Noteworthy in this respect is the fact that Bohus (1977) has demonstrated, using pharmacological methods, that VP might be involved in the maintenance of post-castration male copulatory behavior.

Another indication that centrally acting VP is involved in reproductive processes is given by its inhibitory effect on female sexual behavior after intracerebroventricular administration of VP (Södersten et al., 1983; Södersten, 1984). Although it is not known via what mechanism VP exerts this action, the substrate could be constituted by the sexually dimorphic innervation of the lateral septum, especially since various studies have demonstrated that the septal nuclei are implicated in the (predominantly) inhibitory control of

feminine sexual receptivity (Nance et al., 1974; Zasorin et al., 1975; McGinnis and Gorski, 1976; Gorzalka and Gray, 1981; Nance, 1982). The question of whether or not VP is indeed implicated in the central regulation of male and female sexual behavior and, if so, by which part of the VP innervation, might be studied, e.g., by means of local administration of VP agonists and antagonists in specific brain regions.

Besides the data obtained from the aforementioned pharmacological studies also comparative studies are in support of a role for VP in reproductive functions. Non-mammalian vertebrates do not synthesize the neurohypophyseal hormone VP, the homologue hormone in these classes being vasotocin (Pickering, 1978). Several studies have demonstrated that in many species vasotocin is implicated in reproductive processes (for review see Moore and Miller, 1983). The effects of central injection of vasotocin and antagonists or vasotocin antisera in the newt indicate that vasotocin stimulates male sexual behavior at the level of the brain (Moore and Miller, 1983). That vasotocin and vasopressin may be involved in similar central processes is suggested by a recent finding of Stoll and Voorn (1983) who reported that the vasotocin innervation of the lateral septum of *Gecko gekko*—a lizard species—displays similar sex differences as does the VP innervation in the rat.

### Concluding Remarks

Studies of the vasopressinergic innervation of the brain have demonstrated that immunocytochemistry in conjunction with tracing and lesion studies forms a powerful tool to study the anatomical background of a particular sex difference. Furthermore, it shows that it might be used to study the influences of gonadal hormones, either in development or in adulthood, upon a specific neurotransmitter system. However, immunocytochemical methods have certain inherent drawbacks. One major problem is that it is difficult to raise an antiserum which is directed specifically against a single antigen, which makes it necessary to extensively test all sera and, if necessary, to purify them (Pool et al., 1983). Furthermore, once a section has been stained immunocytochemically, the absence of staining does not exclude the presence of the antigen against which the serum was raised, as has been discussed above. An advantage of the application of immunocytochemistry is that it can readily be combined with other techniques, e.g., with steroid receptor labeling as mentioned previously (Sar and Stumpf, 1980; see also Harlan et al., 1984), with retrograde tracing techniques (Sofroniew and Schrell, 1981; Sawchenko and Swanson, 1982), and even with electrophysiology for identifying the cells recorded from (Skirbol et al., 1981). This

approach enables the simultaneous study of several features of one and the same cell. To conclude the study of the neurotransmitter content of the various components of a given sexually dimorphic system (in addition to, e.g., electrophysiological studies, steroid implantation studies, lesion studies [reviewed by Gorski, 1984; Dyer, 1984; Harlan et al., 1984]) can also help to elucidate its functional significance, if only because the knowledge about the neurotransmitters involved makes it possible to use pharmacological tools such as local administration of specific agonists or antagonists.

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## Discussion

- K. D. Döhler:* Concerning a possible function for the sexual dimorphism of the vasopressinergic innervation of the lateral septum; may it be more than just a coincidence that Nance et al. (1975) induced lordosis behavior in male rats after lesioning the lateral septum, whereas Södersten et al. (1983) inhibited lordosis behavior in female rats after treatment with vasopressin? May this not indicate that the lateral septum and/or vasopressin in this area are inhibitory for lordosis behavior?
- G. J. de Vries:* Lesioning of the lateral septum increased indeed estrogen-induced feminine sexual behavior in the female. In male rats, however, an increase was found only after chronic estrogen treatment following surgery with estrogen (Nance et al., 1975). Another confusing fact in this respect is, that while the lateral habenular nucleus shows a similar sex difference in the VP innervation, lesions of this structure reduce female sexual behavior (Modianus et al., 1974).
- P. Södersten:* Lesions in the lateral septum have been shown to facilitate female sexual behavior in female rats. Can the VP innervation of the lateral septum be important in this? Probably not, since the VP innervation in the lateral septum disappears after castration of male rats and this does not facilitate female sexual behavior in the male.
- G. J. de Vries:* Together with VP many other neurotransmitter systems show changes after gonadectomy (see this chapter). This obviously makes it very difficult to relate changes or the absence of changes in behavior after gonadectomy to the effects observed in one single neurotransmitter system. It therefore seems preliminary to exclude that the VP innervation of the lateral septum exerts an inhibitory influence on female sexual behavior.
- C. D. Toran-Allerand:* Do you have any evidence as to whether or not the loss of immunoreactivity in the castrated adult male is due to loss of fibers or to loss of product?
- G. J. de Vries:* No we have not. Taking into account, however, that DeVoogd and Nottebohm (1981) have shown that steroids can induce neurite outgrowth in the adult bird brain one should not exclude that changes in neuritic length under influence of steroid hormones could also occur in the adult mammalian brain which might include VP fibers as well.
- R. Dyer:* Following the line of reasoning of the last question I would like to know whether there are any circumstances where physiological stimuli can be used to change the density of immunohistochemically stained VP fibers. What happens for example to the density of staining when rats are salt-loaded? This stimulus will activate the VP release system.
- R. Ravid:* Water deprivation causes significant changes in the VP content and distribution in the rat brain (Epstein et al., 1983). Radioimmunoassay shows that VP decreases in the hypothalamus, thala-

mus, septum, striatum, and amygdala. Pituitary VP decreases too, while plasma VP rises. Immunocytochemical staining demonstrates a decrease in VP immunoreactivity in perikarya of the PVN and SCN, while magnocellular fibers are more pronounced due to the presence of large Herring bodies. In the thalamus and septum VP immunoreactivity is very much reduced. This reduction appeared as a loss of immunoreactivity per individual fiber. A clear change in fiber density was, however, not noted.

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## MECHANISMS FOR CREATING DIMORPHISMS: IF THERE ARE SEXUAL DIMORPHISMS... HOW DO THEY GET THAT WAY?

Studies in the previous section relied on the androgen/estrogen–deprivation/replacement paradigm assuming that all sexual dimorphisms are established by hormonal influence during a critical period for sexual differentiation.

The groundbreaking studies in part III investigate the underlying actions of steroid hormones, genes, and experience in creating difference. They contribute to our basic understanding of the action of steroid hormones and how that action is mediated. Genetic studies raise the suggestion that some dimorphisms may be established before androgens and estrogens are even produced and, thus, that some sexual dimorphisms may be the result of chromosomal action that is independent of SRY. With new methods for studying this possibility being developed, as this book goes to press, a new and paradigm-shifting area of research is currently in development.

As the studies in part III reveal, the action of steroid hormones is not simple; it can be mediated by a variety of steroid receptors—classical and nonclassical—it can be activated by gene expression, or activate gene expression, as well as be activated by experience.

Based on the work presented here, it is worth considering how many other estrogen receptors might be as yet undiscovered—ER beta was only uncovered in the late 1990s and ER-X is still highly controversial. There are as yet no variations on the classical androgen receptor, but it has not undergone the same intense scrutiny as the estrogen receptor. One might also admire the intertwining of nature and nurture with the role experience plays in switching on hormone systems. Ask yourself how relevant the notions of organizational and activational are for the female central nervous system—in fact, these studies may bring into question the whole notion of a “critical period” for the female central nervous system. Finally, if you take anything away from this section, let it be that androgens and estrogens are not “sex” steroids—they are growth factors that work both independently and in tandem with the classical growth factors like nerve growth factor and brain-derived nerve growth factor.

Reading the papers in this section will provide insight into

1. The multiplicity of steroid receptors and their relative distribution and action in the brain;
2. Estrogens as growth factors and their ability to make neurons more active and more connected;
3. The phasic growth of connectivity with the rise and fall of estrogens over the ovulatory cycle;
4. A role for genes (other than those for steroid hormones) in establishing sexual dimorphisms; and
5. The possibility that a fundamental and not well-explored sex difference is that androgens/estrogens early in development (as is the case for males) may lead to less plastic neural networks while androgens/estrogens late in development (as is the case for females) may lead to more plastic neural networks.

### Receptor-Mediated Estrogenic Effects

The papers in this section describe experiments designed to understand where and how androgens and estrogens are acting. Some use the now mostly outdated method of radioactively labeling the substance whose receptors they wish to identify. Others use molecular techniques to demonstrate the presence of the receptor itself. All the selections represent critical links in our understanding of where in the brain steroid hormones might act. This constitutes an important first step in any understanding of the effects of hormones on tissue.

Sar and Stumpf were two of the first investigators to document the presence and distribution of androgen receptors in the mammalian brain. Their study shows that androgen receptors are present in the regions that are dimorphic between female and male. Interestingly, although sparse, there are also androgen receptors distributed to cognitive regions (neocortex) as well as to regions directly involved in the production and release of gonadal steroids (pituitary). This study provides evidence that androgens may act directly to organize the male brain without being metabolized to estrogen. Remember that “rough and tumble play” is actually one

behavior for which androgens, not estrogens, are necessary.

Shughrue and colleagues' study on the changing distribution of estrogen receptors during development suggests that, just like the other members of the steroid thyroid superfamily of receptors, differential timing of the appearance of estrogen receptors mediates different developmental stages in the lives of cerebral cortex neurons. It reminds us that the organizational period for sexual differentiation is mediated by the same types of mechanisms as other events in neural development.

Kudwa and colleagues investigate the hypothesis that the classic estrogen receptor, ER alpha, and the more recently discovered estrogen receptor, ER beta, play different roles in shaping the male central nervous system. Readdressing the question of the different stages of the male brain's development they hypothesize that ER alpha is responsible for "masculinization" and ER beta is responsible for "defeminization". Using genetically engineered mice, they are able to study the effects of the presence and lack of ER beta on mouse behavior.

Toran-Allerand and her colleagues present what are perhaps the most controversial experiments within this reader. True to her continually paradigm-shifting work, Toran-Allerand proposes that there is a new, non-nuclear, plasma membrane-associated estrogen receptor, which she calls ER-X. In her own words:

We have shown that the estrogen receptor responsible for mediating estrogen activation of the MAPK cascade is neither the classical receptor ERalpha nor ERbeta. ([http://www.cumc.columbia.edu/research/Faculty\\_Profiles/profiles/toran-allerand\\_cd.html](http://www.cumc.columbia.edu/research/Faculty_Profiles/profiles/toran-allerand_cd.html))

Read these selections for a better understanding of the first principles of steroid hormone action—that it most often takes place via nuclear receptors that have a particular distribution throughout the mammalian brain. Note, however, that the full compendium of estrogen and most likely, androgen, receptors may not yet be written. There has only been a recent unveiling of estrogen receptor beta showing it to have mostly a nonoverlapping distribution as well as function to the classic estrogen receptor alpha. In addition, there is a continuing search for non-nuclear receptors to explain estrogen's sometimes rapid effects on behavior and cell signaling. This had led to the discovery of ER-X, a plasma membrane-associated receptor that appears and disappears at different stages of development and illness. All of these experiments link steroid hormone action with classic neural developmental events that require cell signaling and differential timing to establish the migration and identity of neurons and the development of functional brain regions.

### Estrogens and Growth Factors

The action of steroid hormones through nuclear and potentially plasma-membrane receptors represents one of the major players in sexual differentiation; however, what is it that estrogens *actually* do? The papers in this chapter describe how estrogens both are growth factors and act in tandem with growth factors to modify the genetic expression of neurons in many different regions of the mammalian brain.

In her 1976 paper, Dominique Toran-Allerand was the first to demonstrate the remarkable actions of estrogens on neurons. In her groundbreaking experiments, using hypothalamic explants she showed that the addition of estrogen to the bath led to a profusion of neurite outgrowth rivaling the now famous figure of Rita Levi-Montalcini that showed the effects of nerve growth factor on dorsal root ganglion neurons. In fact, publication of the *Brain Research* paper was difficult because the reviewers could not be convinced that nerve growth factors *were not* in the bath.

Riesert and her colleagues subsequently demonstrated that neurons in the mesencephalon (midbrain) were also sensitive to the growth factor-like effects of steroid hormones. Her paper, which includes Toran-Allerand in the authorship, implicates neurons that secrete tyrosine hydroxylase, a precursor to dopamine, in brain circuits that are sensitive to the action of steroid hormones.

Toran-Allerand and colleagues continued to tease apart the action of estrogens and in this paper report on the colocalization of growth factor receptors with estrogen receptors, a geographical correlation that allows growth factors and steroid hormones to interact synergistically. This study demonstrates yet another brain region where steroid hormones have their effect, the basal forebrain. In addition the experiment reveals that it is cholinergic neurons that are responsive to steroid hormones, leading to speculation that, because this system is implicated in Alzheimer's disease, a reduction in steroid hormone action might play a role in the degeneration of these neurons in Alzheimer's disease (AD).

Read these papers for their surprising demonstration that estrogens are not just about sex—but about growth. Suddenly the effect of estrogen supplementation on the spread of breast cancer becomes clear. Notice that these studies use time-honored cell biological methods to observe estrogenic effects in cell culture as well as establish their interactions with growth factor receptors. The cellular and molecular actions of estrogens has become big business as drug companies search for more effective treatments for the symptoms of both surgical and natural menopause. These selections also

demonstrate that estrogens may be an important mechanism to stave off neuronal degeneration in AD as well as in normal aging.

### Estrogens and Plasticity

Where there is growth, there is plasticity. Selections in this chapter show how estrogens act on neurons to alter size, number, and density of synapses in brain regions as expected as the ventral medial nucleus of the hypothalamus and as unexpected as the CA1 region of the hippocampus.

Jones and colleagues show that after administration of estrogens some of the earliest changes are in the neuron's nucleus leading to increased size and density of chromatin indicating transcription.

Frankfurt and McEwen demonstrate that the density of synaptic spines can be altered on neurons in the ventral medial nucleus.

Woolley and McEwen show that estradiol mediates a "natural" increase and decrease of spines on CA1 pyramidal neurons over the course of the four day ovarian cycle in the female rat.

Read these papers for their very beautiful anatomy and their surprising demonstration that neurons can alter their synaptic connections cyclically and as frequently as every 4 days. Note that these changes are mediated by such standard cell biological processes as increasing protein synthesis and altering gene expression. It makes one realize that, at least for the female rat, change is a constant.

### Genes

Into the story of hormones regulating sex differences through both organizational and activational mechanisms as established by Beach, Young, and Goy emerges the possibility that some sex differences are established earlier than the critical period for sexual differentiation. Currently, there is little evidence supporting this mechanism, but genetic mechanisms have become a very exciting area of research and are being pursued by several groups. It is currently unclear whether these genetically induced differences are due to having two X chromosomes with reliance on the expressed region of the unexpressed X to contribute two copies in females, on the Y chromosome, or on autosomes. The story is currently unfolding.

Beyer and colleagues were the first to suggest that sex differences in the brain might be independent of hormonal effects. Their 1992 report details the finding that a sex difference in the density of prolactin cells in the hypothalamus exists *before* the testes begin to secrete testosterone. They suggest the possibility of genetic mechanisms.

De Vries and colleagues describe a model system that allows the teasing apart of hormonal and genetic mechanisms in establishing sex differences. Their model is a mouse that can be genetically altered to either carry two X chromosomes, one of which also carries the SRY portion of the Y chromosome, or an X and a Y chromosome with the Y chromosome denuded of its SRY portion. This tremendously exciting model will enable us to begin to study even the relative influence of steroid hormones on the progression of diseases that are more common in women such as autoimmune conditions and AD. Imagine the different kinds of double transgenics and what they could show us about sex differences in health.

Read these selections for the elegant way in which they demonstrate sex differences before birth and then use molecular biological techniques to follow these differences after birth. One question arises concerning how big these genetic differences really are in the face of the major changes made by steroid hormones. As well, one might ask, "How much of a behavioral effect do they really have?" Whatever the eventual answer to that question these experiments convince that some brain differences between XX and XY organisms may be established before society has the opportunity to drive them by cultural expectations.

### Experience

Finally, no consideration of malleability would be complete without experience as an agent for sculpting differences. Who of us would be bold enough to assert that XX and XY individuals live the same lives? In human societies these different lives and the different sociopolitical circumstances in which we find ourselves as women and men, themselves can shape sex differences. It is critical to not lose sight of the fact that these act on the brain in the form of learning just as surely as do hormones and genes. In fact, they can influence the production of steroid hormones and gene expression, thereby engaging multiple pathways of influence. To highlight experience as a mechanism for creating sexual dimorphisms, this chapter contains papers that investigate the sculpting of the female nervous system by the experience of child bearing and rearing.

In the first reading, McEwen posits that there is no real separation of nature and nurture anymore—that, in fact, nurture can sculpt nature through hormonal systems. He makes the point that, in contrast to earlier beliefs, sensory receptors are not the only mechanism by which the world makes its way into the brain; steroid hormones are another mechanism. In this chapter, McEwen lays out the evidence for that view.

Modney and Hatton's beautiful chapter relates their studies of the effects of parturition and nursing on

the synaptic structure of vasopressin- and oxytocin-secreting neurons in the hypothalamus. Surprisingly, it is not the sensation of nursing that changes the relationship of these neurons to each other but olfactory cues that come from licking the anal regions of the pups; this change can also be induced in virgin rats who lick pups. Just as surprising, the synaptic relationship of these neurons return to their preparturition state once nursing ceases.

Xerri and colleagues tell the now classic story of how experience changes both somatotopic representation and receptive field size. In contrast to most other papers mapping somatosensory cortex, these experiments are carried out on nursing females, treating nursing as somatosensory stimulation. The results are both what you would expect and surprising in that they demonstrate how the most ordinary activities modify our brains.

Read these selections for their wide-ranging implications about how life continues to sculpt the differences that arise even before birth. Note that new methods and models need to be developed continually to follow up on unexpected observations. If one finds a sexual dimorphism now it will not be sufficient to apply the earlier paradigm of organizational and activational effects and the influence of steroid hormones. The difference may have been established by genetic mechanisms even before the critical period. Sex differences also may be established long after the critical period and by the more common mechanisms associated with learning and memory. Finally, note that the findings described in this section deeply trouble the notion of a critical period—since mechanisms for change are acting throughout life—perhaps especially in women.

## Introduction

Actions of testosterone on the regulation of gonadotropin secretion and sexual behavior in male animals have been thought to be mediated through metabolites rather than the hormone itself. This is supported by the finding that testosterone can be metabolized to androstenedione (4-androstene 3,17-dione) and 5 $\alpha$ -dihydrotestosterone or aromatized to estrogen by brain tissue [1–4]. Substantial amounts of DHT, androstenediol and testosterone have been extracted from rat brain following infusion of [ $^3\text{H}$ ]-testosterone [5]. After [1,2- $^3\text{H}$ ]-testosterone injection, uptake of radioactivity into nuclei of neurons in hypothalamic and extrahypothalamic sites, as well as in anterior pituitary cells has been demonstrated [6–8]. Since estrogen can be produced from testosterone in the brain, the notion exists that all or some of the central actions of testosterone are mediated through estrogen rather than androgen [9].

This report provides further evidence of androgen target cells in the forebrain and pituitary after injection of [ $^3\text{H}$ ]-dihydrotestosterone (DHT), a non-aromatizable androgen, with or without prior administration of estradiol-17 $\beta$  as potential competitor, using autoradiographic techniques developed in our laboratory for the study of non-covalently bound compounds [10].

## Materials and Methods

Six 26-day-old and three 60-day-old male Holtzman rats, orchietomized and adrenalectomized for 96 h, were each injected intravenously with 1 or 2  $\mu\text{g}$  per 100 g body weight of [1,2 $\alpha$ - $^3\text{H}$ ]-dihydrotestosterone, S.A. 44 Ci/m-mol (New England Nuclear), dissolved in 10% ethanol in isotonic saline. The radiochemical purity was established by thin layer chromatography using the solvent system of benzene–ethyl acetate (2:1, v/v) and found to be greater than 98%. In order to examine whether or not estradiol can inhibit the nuclear uptake of radioactivity after [ $^3\text{H}$ ]-dihydrotestosterone injection, two immature rats were each injected intra-

venously 5 min prior to the labeled DHT with 2  $\mu\text{g}$  or 20  $\mu\text{g}$  per 100 g body weight of estradiol-17 $\beta$ , dissolved in ethanol. In addition, two orchietomized and adrenalectomized immature rats were injected intravenously, each with 100  $\mu\text{g}$  of unlabeled DHT per 100 g body weight 5 min prior to the injection of [ $^3\text{H}$ ]-DHT, to show the specificity of androgen localization.

One hour after injection of [ $^3\text{H}$ ]-dihydrotestosterone, the rats were killed, the brain and pituitary removed, mounted on tissue holders, and frozen in  $-180^\circ\text{C}$  liquefied propane. Four  $\mu\text{m}$  serial frozen sections were cut in a Wide Range Cryostat (Harris Mfg. Co., North Billerica, MA) and dry- or thaw-mounted on photographic emulsion (Kodak NTB-3) coated slides. The brain tissues and pituitary from an untreated orchietomized and adrenalectomized rat were also processed as control for chemographic artifacts. After autoradiographic exposure for 4–9 months, the slides were photographically processed and stained with methylgreen pyronin for DNA and RNA [10].

## Quantification

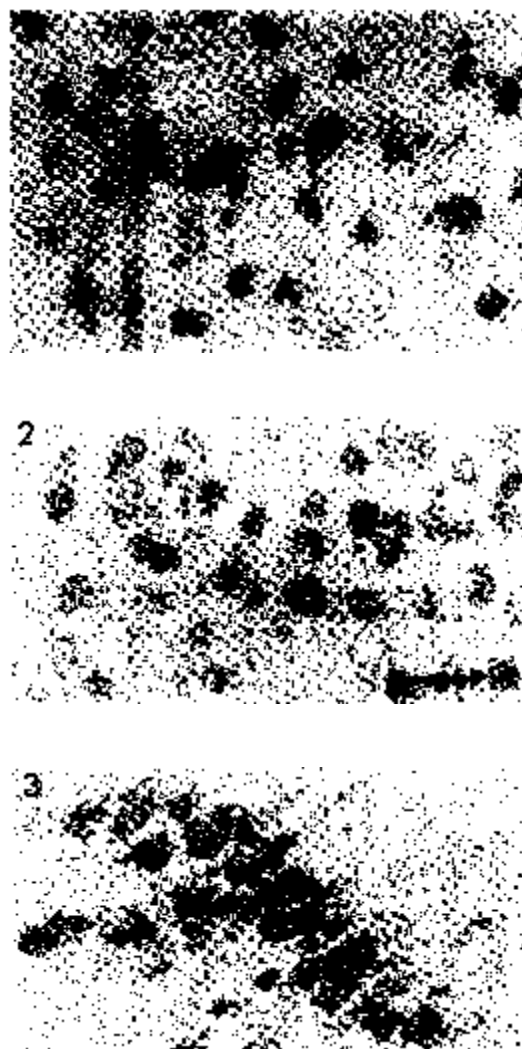
The average number of silver grains in nuclei of neurons was determined by counting the number of reduced silver grains in 50–100 labeled neurons of a specific nucleus, using 100 $\times$  objective, after correcting for background radioactivity. The background radioactivity was determined by assessing the number of silver grains per square  $\mu\text{m}$  in areas not occupied by tissue sections. Assuming that nuclei of neurons are round, the nuclear area was expressed in square  $\mu\text{m}$  and the background radioactivity per nucleus was calculated by multiplying the number of background silver grains per unit area with the total nuclear area. The value for nuclear concentration of radioactivity was obtained by subtracting the background radioactivity from total radioactivity. The student t-test was utilized to determine the significant level between control and estrogen treatment groups. Each labeled neuron of a particular hypothalamic nucleus was considered as one sample when significance levels were determined between identical hypothalamic nuclei of different groups. A cell was considered labeled when

the nucleus contained four or five times the number of silver grains per unit area above background.

## Results

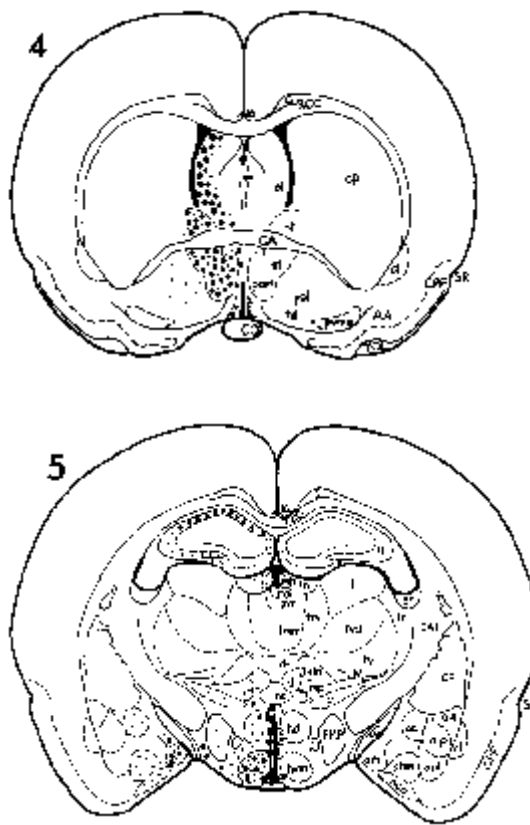
Autoradiograms of brain tissue of orchietomized and adrenalectomized immature and mature male rats show concentration and retention of radioactivity in nuclei of certain neurons and ependymal and subependymal cells in certain regions, as well as cells of the pia mater (figures 42.1–42.3). The topographic distribution of the labeled cells appears similar in immature and mature rats (figures 42.4 and 42.5). In the area of the lamina

terminalis radioactively labeled neurons are found ventrally in the nucleus (n.) tractus diagonalis and in the region of the optic recess organ, and dorsally in the n. septi lateralis (figures 42.1 and 42.4) and n. triangularis septi. In the hippocampus neurons in the pyramidal layer show radioactive labeling throughout (figure 42.3), with neurons in CA1 and CA2 displaying stronger accumulation of radioactivity when compared with CA3, CA4 and subiculum, while under the same conditions, neurons of the dentate gyrus are not labeled (figure 42.5). In the amygdala, neurons of the n. amygdaloideus medialis are strongly labeled in contrast to



**Figures 42.1–42.3**

Autoradiograms of rat prepared 1 h after intravenous injection of [ $^3$ H]-dihydrotestosterone into orchietomized and adrenalectomized rats showing nuclear concentration of radioactivity in neurons of nucleus septi lateralis (figure 42.1), in neurons of nucleus preopticus medialis (figure 42.2), and in pyramidal cells of hippocampus (figure 42.3). Exposure time 270 days. Magnification  $\times 520$ . Stained with methyl-green pyronin. cp is nucleus caudatus putamen; v is ventricle.



**Figures 42.4 and 42.5**

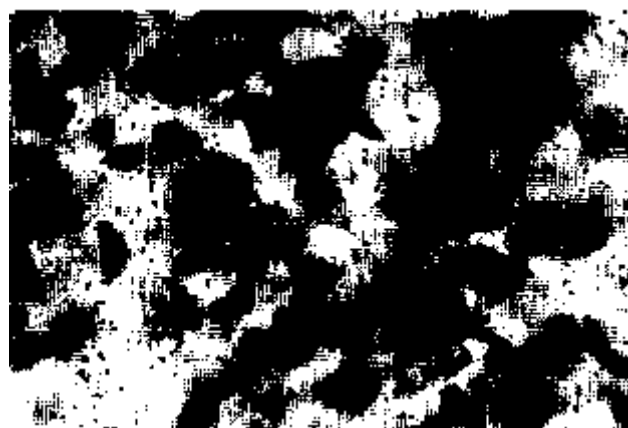
Schematic drawings showing distribution of androgen concentrating neurons in preoptic region (figure 42.4) and in central hypothalamus (figure 42.5), in frontal plane 1 h after injection of [ $^3$ H]-dihydrotestosterone. The size and number of dots indicates the intensity of nuclear uptake of radioactivity and the frequency of occurrence of androgen concentrating cells. The schematic drawings were prepared after serial section autoradiograms according to the atlas of König and Klippel [23]. Abbreviations: abm, nucleus (n) amygdaloideus basalis pars medialis; ac, n. amygdaloideus centralis; ace, n. amygdaloideus corticalis; am, n. amygdaloideus medialis; ar, n. arcuatus hypothalami; CA, Commissura anterior; CO, chiasma opticum; FMP, Fasciculus medialis prosencephali; FO, Fornix; hd, n. dorsomedialis hypothalami; HI, hippocampus; hpv, n. periventricularis hypothalami; hvm, n. ventromedialis hypothalami; mh, n. medialis habenulae; pol, n. preopticus lateralis; pom, n. preopticus medialis; posc, n. preopticus pars suprachiasmatica; sl, n. septi lateralis; st, n. interstitialis striae terminalis; zi, zona incerta. For other abbreviations see König and Klippel [23].

the weakly labeled neurons of the n. amygdaloideus centralis, n. basalis pars medialis, and n. corticalis (figure 42.5). In the preoptic area, labeled neurons are concentrated in the n. preopticus medialis and lateralis (figures 42.2 and 42.4). Accumulations of labeled neurons exist further in the n. interstitialis striae terminalis, n. periventricularis hypothalami, n. paraventricularis, n. ventromedialis, n. arcuatus and n. premammillaris ventralis (figure 42.5). Dispersed labeled neurons are also seen in the n. dorsomedialis, the n. perifornicalis and the zona incerta, the anterior, lateral and posterior hypothalamic areas, further in the n. premammillaris dorsalis, n. prelateralis mammillaris, n. supramammillaris, n. mammillaris posterior, n. parafascicularis and the periventricular gray. Certain cells of the organum subfornicale, n. habenulae medialis and pineal are labeled. Cells of the cerebral cortex do not appear to be labeled under the present experimental condition.

In the anterior pituitary only a small percentage of cells (approximately 10–15%) are labeled after injection of [ $^3\text{H}$ ]-DHT (figure 42.6). These cells are identified as gonadotropes by immunocytochemical staining using antisera against ovine LH or its  $\beta$ -subunit (unpublished observation). The results are comparable to those obtained with [ $^3\text{H}$ ]-testosterone [7, 8].

Differences in the intensity of neuronal nuclear labeling exist among different nuclear areas of the brain (table 42.1). For instance, the intensity of subcellular nuclear labeling in neurons is highest in the nucleus (n.) septi lateralis, n. premammillaris ventralis, and n. periventricularis hypothalami; it is medium in neurons of the n. interstitialis striae terminalis, n. preopticus medialis and lateralis, and n. arcuatus hypothalami; and it is medium to low in neurons of the n. ventrome-

dialis hypothalami, n. medialis amygdalae and the pyramidal layer of the hippocampus (table 42.1). Nuclear concentration of radioactivity in neurons is inhibited when unlabeled 5 $\alpha$ -DHT was injected before the injection of [ $^3\text{H}$ ]-DHT. Administration of unlabeled estradiol-17 $\beta$  at a dose similar to the labeled DHT 5 min prior to the injection of labeled DHT has no or little effect on the nuclear uptake of radioactivity in neurons, whereas a dose of estradiol-17 $\beta$  ten times higher than the dose of labeled DHT reduces the nuclear concentration of radioactivity in such areas as n. septi lateralis ( $P < 0.05$ ), n. preopticus medialis ( $P < 0.01$ ) and n. premammillaris ventralis ( $P < 0.01$ ) by 20–25% (table 42.1, figures 42.7–42.9).



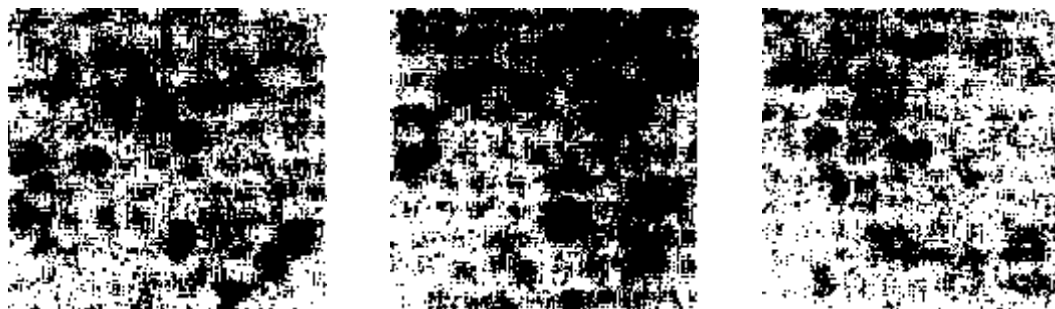
**Figure 42.6**  
Autoradiogram of rat anterior pituitary showing nuclear concentration of radioactivity in a few cells, after intravenous injection of [ $^3\text{H}$ ]-dihydrotestosterone. Exposure time 110 days. Magnification  $\times 520$ . Stained with methylgreen pyronin.

**Table 42.1**

Effects of estradiol-17 $\beta$  on nuclear concentration of radioactivity in neurons of rat brain one h after injection of [ $^3\text{H}$ ]-dihydrotestosterone 2  $\mu\text{g}/100$  g body weight

Structure	Control (2)* (Mean $\pm$ S.E.)	Estradiol-17 $\beta$ (2)* 2 $\mu\text{g}/100$ g b.wt (Mean $\pm$ S.E.)	Estradiol-17 $\beta$ (2)* 20 $\mu\text{g}/100$ g b.wt (Mean $\pm$ S.E.)
N. septi lateralis	21.31 $\pm$ 2.22	19.53 $\pm$ 1.72	16.12 $\pm$ 1.08 <sup>†</sup>
N. interstitialis striae terminalis	17.05 $\pm$ 2.03	15.36 $\pm$ 1.20	13.20 $\pm$ 1.60
N. preopticus medialis	18.09 $\pm$ 1.55	15.90 $\pm$ 1.11	12.57 $\pm$ 0.08 <sup>‡</sup>
N. periventricularis hypothalami	22.00 $\pm$ 2.15	21.18 $\pm$ 2.12	18.50 $\pm$ 1.20
N. arcuatus hypothalam	14.46 $\pm$ 1.29	15.00 $\pm$ 0.54	13.58 $\pm$ 1.67
N. ventromedialis hypothalami	10.82 $\pm$ 2.05	10.25 $\pm$ 0.44	8.89 $\pm$ 0.51
N. premammillaris ventralis	22.90 $\pm$ 1.81	19.60 $\pm$ 1.32	17.05 $\pm$ 1.33 <sup>‡</sup>
N. amygdaloideus medialis	11.14 $\pm$ 0.92	11.00 $\pm$ 0.85	10.23 $\pm$ 0.79
Hippocampus (area CA-1)	10.30 $\pm$ 0.68	9.20 $\pm$ 0.40	8.80 $\pm$ 0.91

\*The number in parentheses indicates the number of animals. Unlabeled estradiol-17 $\beta$  dissolved in ethanol was injected intravenously 5 min prior to the injection of [ $^3\text{H}$ ]-dihydrotestosterone. The mean value for nuclear concentration of radioactivity is based on counting of silver grains in 50–100 randomly selected labeled neurons after correcting for background radioactivity. Exposure time 120 days. S.E.: standard error. Significantly differ from the control, <sup>†</sup> $P < 0.05$ ; <sup>‡</sup> $P < 0.01$ .



Figures 42.7–42.9

Autoradiograms of the nucleus preopticus medialis prepared 1 h after injection of [ $^3$ H]-DHT showing the competitive effects of 2  $\mu$ g (figure 42.8) or 20  $\mu$ g (figure 42.9). Estradiol-17 $\beta$  on nuclear uptake of radioactivity. Exposure time 270 days; thickness 4  $\mu$ m; magnification  $\times$ 520; stained with methylgreen pyronin. Note the reduction of nuclear concentration of radioactivity after 20  $\mu$ g of estradiol-17 $\beta$  (figure 42.9), and the lack of reduction after 2  $\mu$ g of estradiol-17 $\beta$  (figure 42.8) as compared to the control without estradiol-17 $\beta$  pretreatment (figure 42.7).

## Discussion

The localization of radioactivity as demonstrated here is androgen since unlabeled estradiol-17 $\beta$  in similar doses as labeled DHT injected prior to [ $^3$ H]-DHT does not inhibit the nuclear uptake of radioactivity and 5 $\alpha$ -dihydrotestosterone, an A-ring reduced steroid, is not convertible to estrogen. These results are consistent with the biochemical findings of specific 5 $\alpha$ -dihydrotestosterone binding protein in the cytosol fraction of male rat hypothalamus, pineal and pituitary [11–13] and the association of DHT with the nuclear fraction of “hypothalamus” and pituitary [14]. Only a dose of estradiol-17 $\beta$  higher than the dose of [ $^3$ H]-DHT reduces the nuclear uptake of radioactivity in certain nuclear groups. These data suggest that the DHT receptor has some low affinity for estradiol, although further experiments are required in order to establish more precisely the receptor specificity.

The present autoradiographic results for the first time describe the topographical distribution of androgen target cells obtained with [ $^3$ H]-DHT. These data in general agree well with our earlier observation on androgen localization in the brain after injection of [ $^3$ H]-testosterone [6, 15]. However, localization of radioactivity in cells of cerebral cortex was not observed after [ $^3$ H]-DHT. In contrast, weakly labeled cells in certain regions of cerebral cortex have been found after [ $^3$ H]-testosterone [15]. The absence of labeling in cortical cells may be attributed to the use of labeled DHT with low S.A. or the cortical cells may have specific affinity for testosterone binding. The concentrated radioactivity in neurons is likely to be DHT, since the major metabolite recovered from brain tissue 30 min after intravenous injection or 3 h after continuous infusion of labeled DHT is DHT [16, 17] and prior injection of unlabeled DHT reduces the nuclear uptake of radioactivity. Whether or not other metabolites of DHT, such as androstenediol and androstenedione have an effect

on [ $^3$ H]-DHT uptake has not been investigated, although androstenedione has been shown to inhibit the nuclear uptake of radioactivity obtained with [ $^3$ H]-testosterone [15].

The autoradiographic demonstration of androgen-concentration in specific regions and cell types in the brain, as well as in the pituitary, suggests a direct action of DHT on these structures. It further supports the concept that DHT is one of the major active metabolites and important for the central action of “testosterone,” as it has been shown for other androgen-responsive tissues such as prostate and seminal vesicles. Dihydrotestosterone has been found to be more potent than testosterone, suppressing serum LH levels while equipotent to testosterone in suppressing FSH [18] and comparable to testosterone in maintaining mating behavior in hypophysectomized rats [19] and in stimulating sexual behavior in guinea pig [20] and Rhesus monkey [21]. Also pretreatment with 5 $\alpha$ -DHT has been reported to increase pituitary LH release by LH-RH [22].

## Acknowledgements

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A sexual dimorphism in reproductive behavior and gonadotropin secretion has been attributed to differences in the “wiring” of the male and female rodent brain. These attributes are determined during late fetal and early postnatal life, when the hormonal milieu has a permanent organizational effect on the sexually undifferentiated brain (1–8). Estrogen is the major hormone responsible for determining the connectivity of certain neurons, and differences in estrogen levels during perinatal life demarcate the phenotype of the adult rodent brain (9, 10). The enzyme aromatase that converts androgens to estrogens (11, 12) is thought to establish a sexual dimorphism in estrogen levels and, ultimately, the wiring of the brain. When high levels of estrogen are present due to the aromatization of androgens, a male brain develops. Alternatively, low submasculinizing levels of estrogen, due to the absence of testis in females, result in female brain development (9, 10).

Extensive research has elucidated the importance of estrogen in brain development, but little is known about its sites of action during postnatal brain development. Comparison of the 2-day-old rat preoptic, central, and posterior hypothalamic estrogen receptors (13) with those in the adult (14) revealed that the distribution of labeled cells in the early postnate was commensurate with that in the adult, although attenuated. One notable exception was the cortex. The 2-day postnatal cortex contained numerous receptor cells throughout laminae V and VI (13), while only a few labeled cells existed in the adult cortex (14). Additional autoradiographic studies have shown the presence of estrogen target cells in the term fetal and early postnatal cortex (15, 16), but not in preceding age groups (17, 18). Biochemical studies demonstrated that cortical estrogen receptor number increases during the first week of postnatal life, peaks, and then declines with subsequent postnatal development (15, 19, 20).

The present study was designed to provide detailed anatomical information about the existence of estrogen receptor cells in the cerebral cortex during postnatal development and to evaluate changes in estrogen receptor number and topography between birth and postweaning. High resolution thaw-mount auto-

radiography with 11 $\beta$ -methoxy-16 $\alpha$ -[<sup>125</sup>I]iodoestradiol ([<sup>125</sup>I]MIE<sub>2</sub>) was used because this compound has high specific activity and binding affinity for the estrogen receptor (21) and poor affinity for rodent  $\alpha$ -fetoprotein (22). Through the use of this radioiodine labeled estrogen, we were able to reduce the photographic exposure time, while retaining the sensitivity needed for the cellular and subcellular resolution (21–24).

## Materials and Methods

### Animals

ICR mice (Harlan Sprague-Dawley, Birmingham, AL) were maintained in a 14-h light, 10-h dark environment, with free access to tap water and rodent chow. One male proven breeder was housed with three nulliparous females and left overnight. Females were checked in the morning, and those with vaginal plugs were housed separately and marked day 0 of pregnancy. Mothers were allowed to deliver and care for pups until the neonates reached the appropriate age. Only mothers with a 19-day gestation were used for experimental groups, and day 0 postnates were used within 10 h after birth.

### Autoradiography

On neonatal days 0, 2, 8, 12, 18, and 25, six mice (three males and three females) were each sc injected in the dorsal cervical region with 0.25  $\mu$ g/100 g BW [<sup>125</sup>I]MIE<sub>2</sub> (SA, 2200 Ci/mmol) dissolved in 20% ethanol-isotonic saline. Radioiodine labeled estrogen was prepared as described previously (21) and used within 7 days of synthesis. For competition, two additional 2-day-old males were each injected with 250  $\mu$ g/100 g BW unlabeled 17 $\beta$ -estradiol (Sigma Chemical Co., St. Louis, MO) 1 h before [<sup>125</sup>I]MIE<sub>2</sub> to show the specificity of [<sup>125</sup>I]MIE<sub>2</sub> for the estrogen receptor. Injection sites were sealed with Silastic medical adhesive (Dow Corning, Midland, MN) to prevent seepage. The pups were then maintained in a infrared heated chamber. Two hours after the injection of [<sup>125</sup>I]MIE<sub>2</sub>, brains were removed and placed on brass mounts with minced liver as an adhesive. Specimens were slowly immersed and frozen in liquid propane (–180 C), then

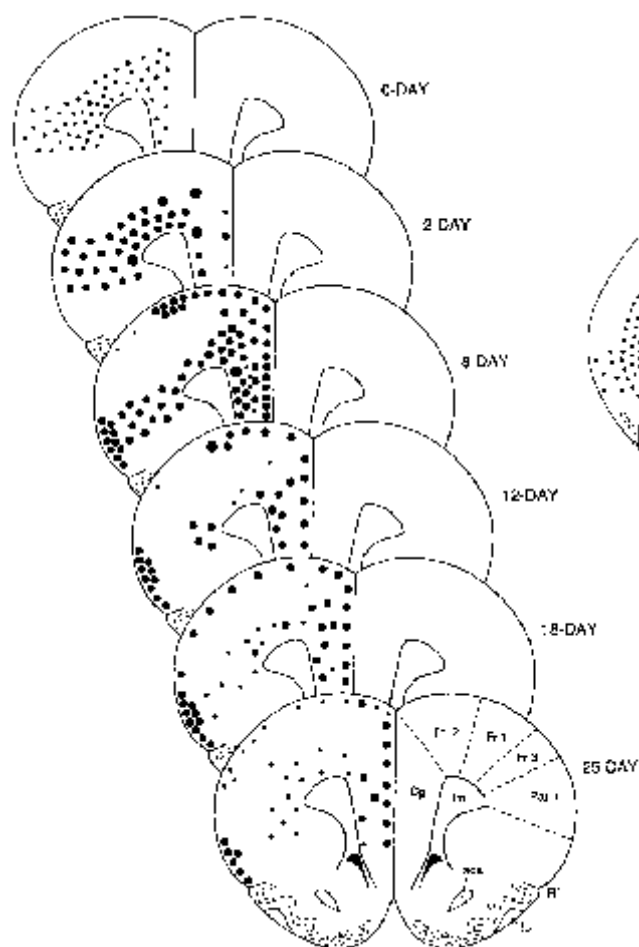


Figure 43.1

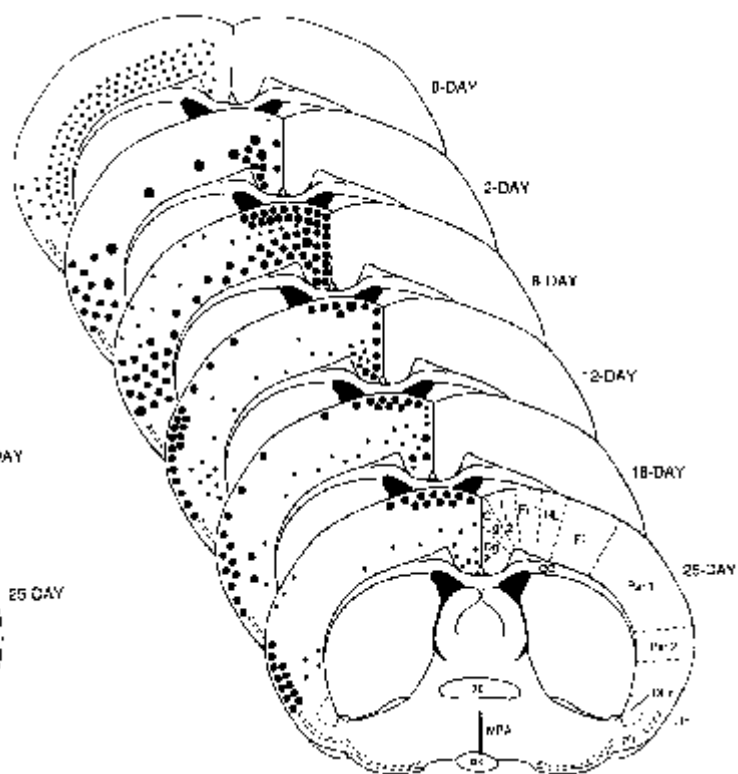


Figure 43.2

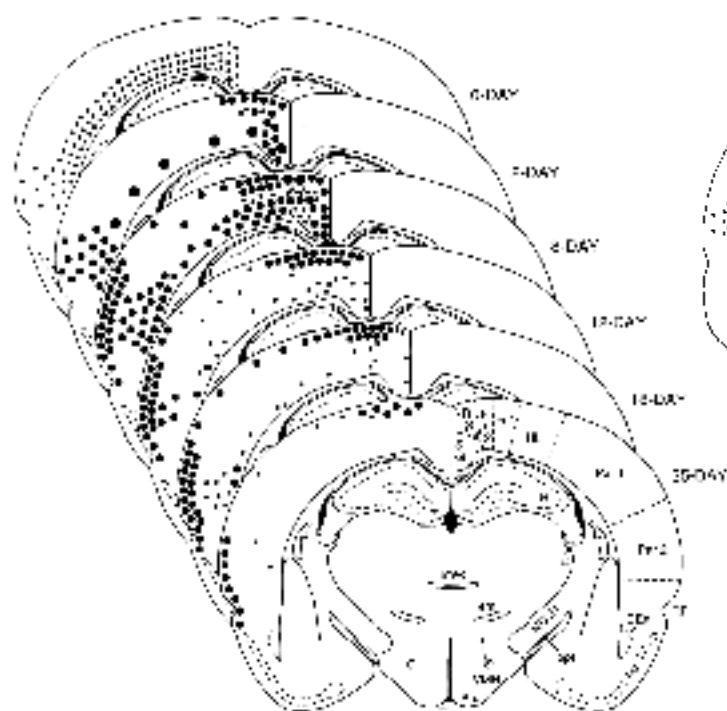


Figure 43.3

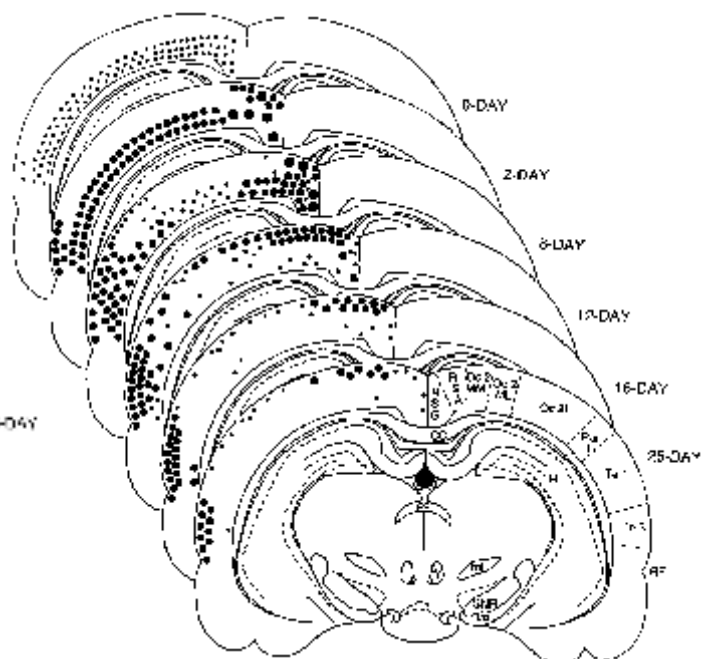


Figure 43.4

stored in liquid nitrogen for a period not exceeding 14 days. Four-micron frontal sections were cut at  $-30^{\circ}\text{C}$  in a cryostat (Reichert-Jung, Cambridge Instruments, Buffalo, NY), thaw-mounted onto photographic emulsion (Kodak NTB3, Eastman Kodak, Rochester, NY)-coated slides, and stored in light-tight desiccator boxes at  $-15^{\circ}\text{C}$ . After 1–45 days of exposure slides were warmed to room temperature, immersed in 4% paraformaldehyde solution for 30 sec, washed, developed in 1:1 Kodak D19 developer for 1 min, washed, and fixed in Kodak fixer for 5 min. Subsequently, these slides were washed in water, stained with methyl-green pyronine, and coverslipped. All processing solutions and washings were maintained at  $15^{\circ}\text{C}$ . Several slides exposed for long periods were coverslipped without staining for darkfield microscopy. Additional details concerning slide and tissue preparation, sectioning, and processing have been previously described (25).

### Quantitative Analysis and Mapping

Autoradiograms were scanned at low magnification using an Olympus microscope (New Hyde Park, NY) to determine the regional distribution of cortical cells, except piriform cortex, concentrating radioactivity at the levels of the frontal pole, preoptic area, and central and posterior hypothalamus. The subcellular distribution of silver grains and laminar dispersement of labeled cells were verified by viewing sections exposed for shorter periods of time at high magnification. Labeled cortical cells were analyzed and mapped at each brain level and for each of the six postnatal age groups. The numerical values obtained from a particular age group were combined so that the representative map of each level was the average of values from the animals in that group. Since no apparent sex differences in the number or distribution of labeled cells was observed, the results from all animals were combined for each map. Dots were then placed onto representative computer scans; their size and distribution represented the quantity of cells with nuclear labeling in a particular region. The laminae and cortical regions were determined with the assistance of cortical maps (26). Since lamination in the 0- and 2-day-old brains was incomplete, extracortical

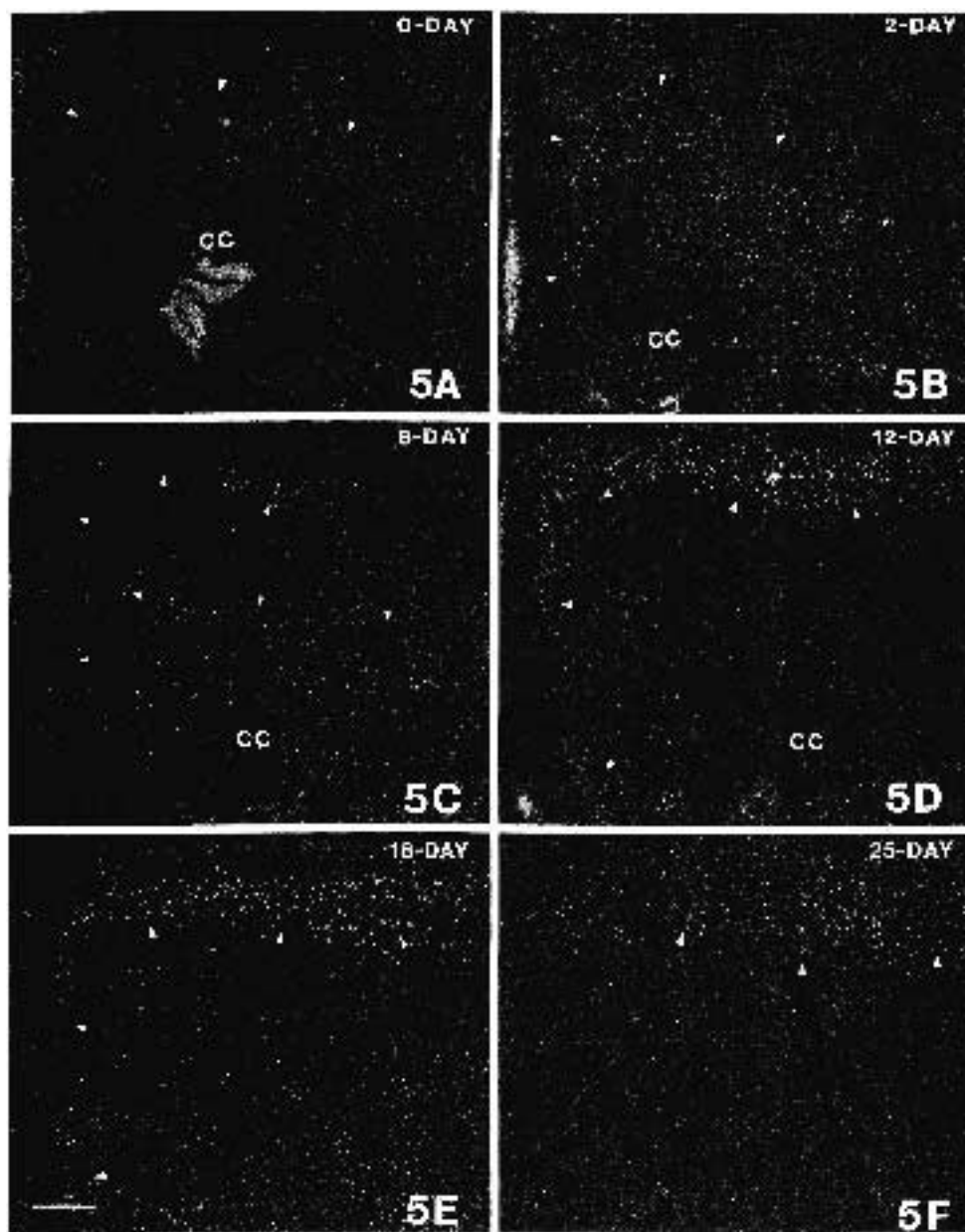
landmarks (i.e., ventricles, hippocampus, etc.) were used to establish regions for mapping, and dots were assigned to deep, intermediate, or superficial layers of the cortex rather than individual laminae. The results obtained from the piriform cortex and noncortical brain regions were evaluated separately.

### Results

Two hours after injection of [ $^{125}\text{I}$ ]MIE<sub>2</sub>, radioactivity was observed throughout the postnatal brain, thus demonstrating that estradiol from an extracranial source was accessible to all parts of the brain. In addition, certain cortical cells had nuclear uptake and retention of radioactivity. The number and topography of cortical estrogen target cells at the levels of the frontal pole, preoptic area, and central and posterior hypothalamic regions have been depicted in four schematic drawings for each age group (figures 43.1–43.4). At birth, labeled cells were found throughout the deep layers, with some weakly labeled cells in the intermediate layer dorsal to the rhinal fissure (suprarhinal cortex). The 2-day-old cortex displayed a dense concentration of labeled cells in the deep cortical layers, with a high number of labeled cells in the deep cingulate region near the interhemispheric cleft. An increase in receptor cell number was also seen in the intermediate and superficial layers of the cortex dorsally and adjacent to the interhemispheric cleft, and ventrally in a confined region of the suprarhinal cortex. By day 8, the number of target cells in the deep cortex (lamina VI) was reduced compared to that in the day 2 brain, and a disproportionate pattern of labeling was observed. Target cells were concentrated in the deep layers of the cingulate/paracingulate and suprarhinal regions, whereas the remaining deep layers between these regions were sparsely labeled. A pronounced increase in cell number and topography was also seen in the superficial (laminae II and III) layers of the dorsal cingulate/paracingulate and the ventral suprarhinal cortex, but not in the intermediate (lamina V) layer. By day 12, the population of labeled cells in lamina V and VI was reduced to a few cells in the cingulate and suprarhinal

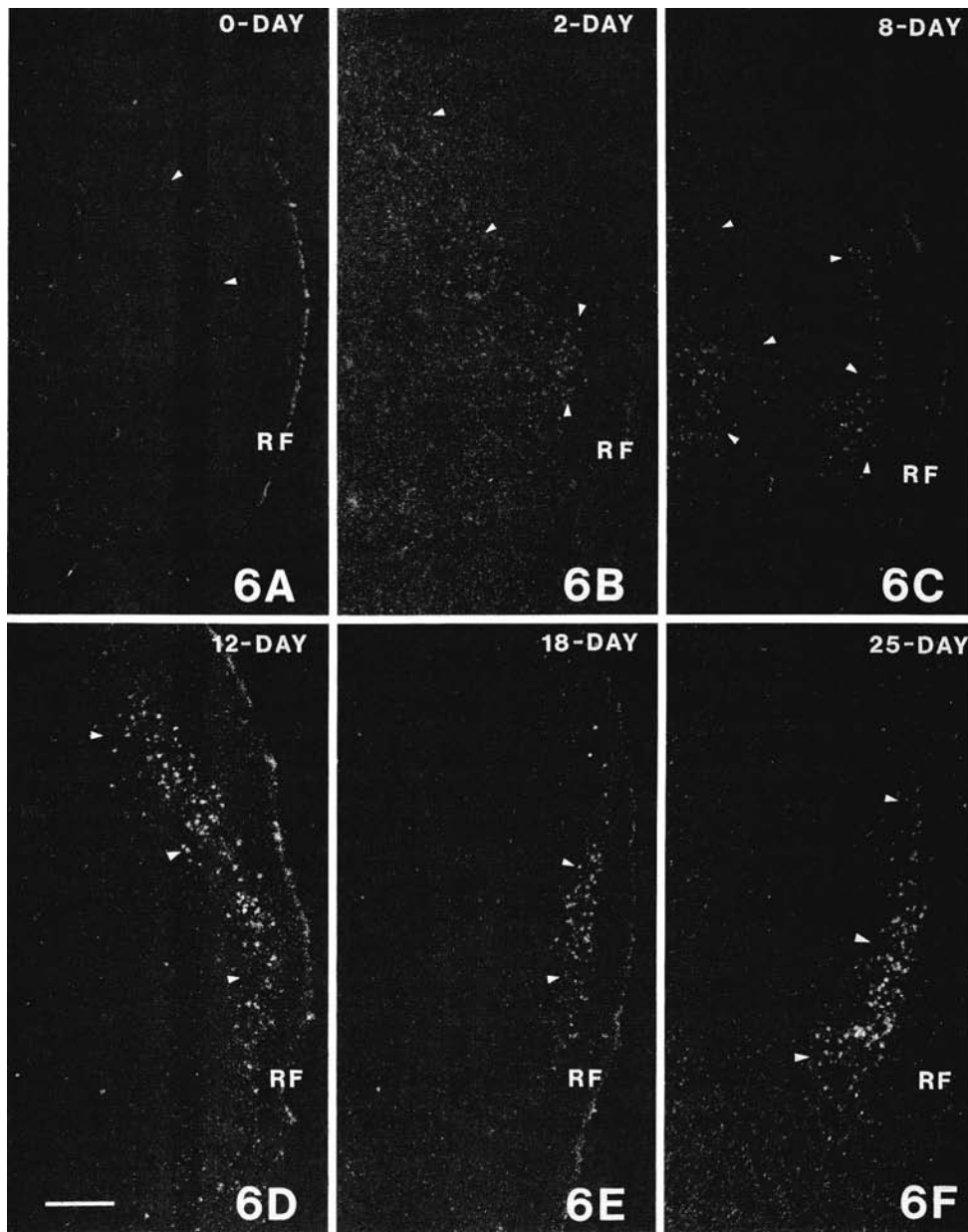
### Figures 43.1–43.4

The distribution of estrogen receptor cells in the 0-, 2-, 8-, 12-, 18-, and 25-day postnatal mouse cortex 2 h after sc injection of [ $^{125}\text{I}$ ]MIE<sub>2</sub>. Schematic drawings generated from autoradiograms of coronal sections, showing the number and topography of labeled cells in the cortex at the level of the frontal pole (figure 43.1), preoptic area (figure 43.2), central hypothalamus (figure 43.3), and posterior hypothalamus (figure 43.4). *Large dot*, 75 labeled cells; *medium dot*, 5 labeled cells; *small dot*, 1 labeled cell. ac, Anterior commissure; aca, anterior commissure, anterior; Arc, arcuate nucleus; CC, corpus callosum; Cg, cingulate cortex; Cg1, cingulate cortex, area 1; Cg2, cingulate cortex, area 2; cp, cerebral peduncle, basal; DEn, dorsal endopiriform nucleus; eml, external medullary lamina; f, fornix; FL, forelimb cortex; fmi, forceps minor corpus callosum; fr, fasciculus retroflexus; Fr1, frontal cortex, area 1; Fr2, frontal cortex, area 2; Fr3, frontal cortex, area 3; HI, hippocampus; HL, hindlimb cortex; imvc, intermedioventral thalamic commissure; lo, lateral olfactory tract; MCLH, magnocellular nucleus lateral hypothalamus; ml, medial lemniscus; MPA, medial preoptic area; Oc2L, lateral occipital cortex, area 2; Oc2ML, mediolateral occipital cortex, area 2; Oc2MM, mediomedial occipital cortex, area 2; opt, optic tract; ox, optic chiasm; Parl, parietal cortex, area 1; Par2, parietal cortex, area 2; pc, posterior commissure; Pir, piriform cortex; RF, rhinal fissure; RSA, retrosplenial agranular cortex; RSG, retrosplenial granular cortex; SNR, substantia nigra, reticular; st, stria terminalis; TEL, temporal cortex, area 1; Te3, temporal cortex, area 3; VMH, ventromedial hypothalamic nucleus. Drawings modified from the atlas of Paxinos and Watson (39).



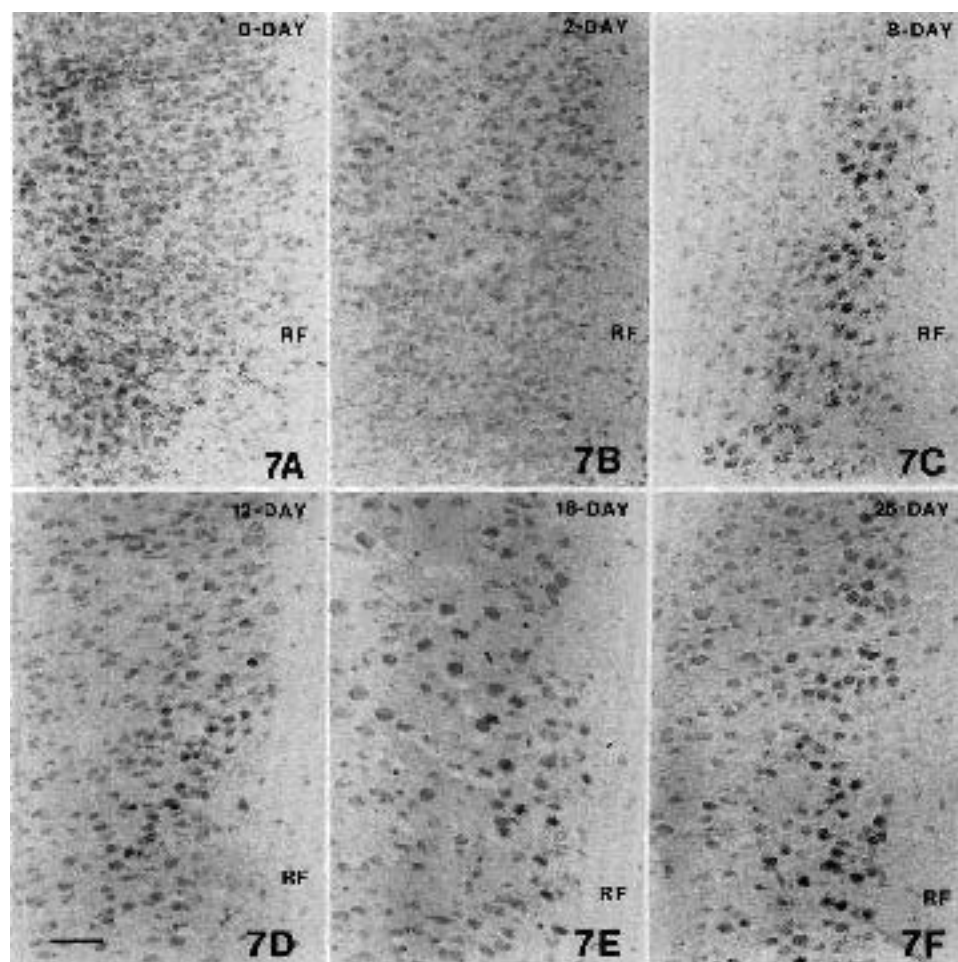
**Figure 43.5**

Autoradiograms of the left cingulate/frontal cortex in the 0-, 2-, 8-, 12-, 18-, and 25-day postnate, at the level of the preoptic area, after sc injection of [ $^{125}$ I]MIE<sub>2</sub>. During postnatal development, labeled cells (*arrows*) in the deep 0-day cortex (A) expanded to the intermediate layer of the 2-day (B) and superficial layers (laminae II and III) of the 8-day cingulate/frontal cortex (C). By day 12, target cells in laminae V and VI were sparse, while the number of labeled cells in laminae II and III of the paracingulate frontal cortex increased (D). Labeling in the paracingulate frontal cortex was attenuated on day 18 (E) and day 25 (F). Radioactivity was also seen in the third ventricle and interhemispheric cleft and on the cortex surface due to ligand in cerebrospinal fluid and blood. Exposure was for 28 days. Magnification,  $\times 64$ . *Bar* = 250  $\mu$ m. CC, Corpus callosum.



**Figures 43.6 and 43.7**

Autoradiograms of the left parietal/suprarhinal cortex in the 0-, 2-, 8-, 12-, 18-, and 25-day postnate, at the level of the preoptic area, after sc injection of [ $^{125}$ I]MIE<sub>2</sub>. During postnatal development, labeled cells (*arrows* in figure 43.6) confined to the deep laminae of the 0-day cortex (A) extend from deep layers to the intermediate and superficial laminae of the 2-day suprarhinal cortex (B). These target cells were concentrated in laminae II–VI of the 8-day parietal/suprarhinal cortex (C), while labeling in laminae V and VI of the 12-day was reduced (D). The degree of labeling in lamina II and III was reduced in the 18-day (E) and 25-day cortex (F). The brightfield photomicrographs in figure 43.7 demonstrate that the concentrations of radioactivity, seen in darkfield photomicrographs of figure 43.6, are due to the uptake and retention of radioactivity by receptor cells. Exposure was for 28 days. Magnification: figure 43.6, 85 $\times$ ; figure 43.7, 200 $\times$ . Bar: figure 43.6, 250  $\mu$ m; figure 43.7, 50  $\mu$ m. Abbreviation: RF, rhinal fissure.



Figures 43.7

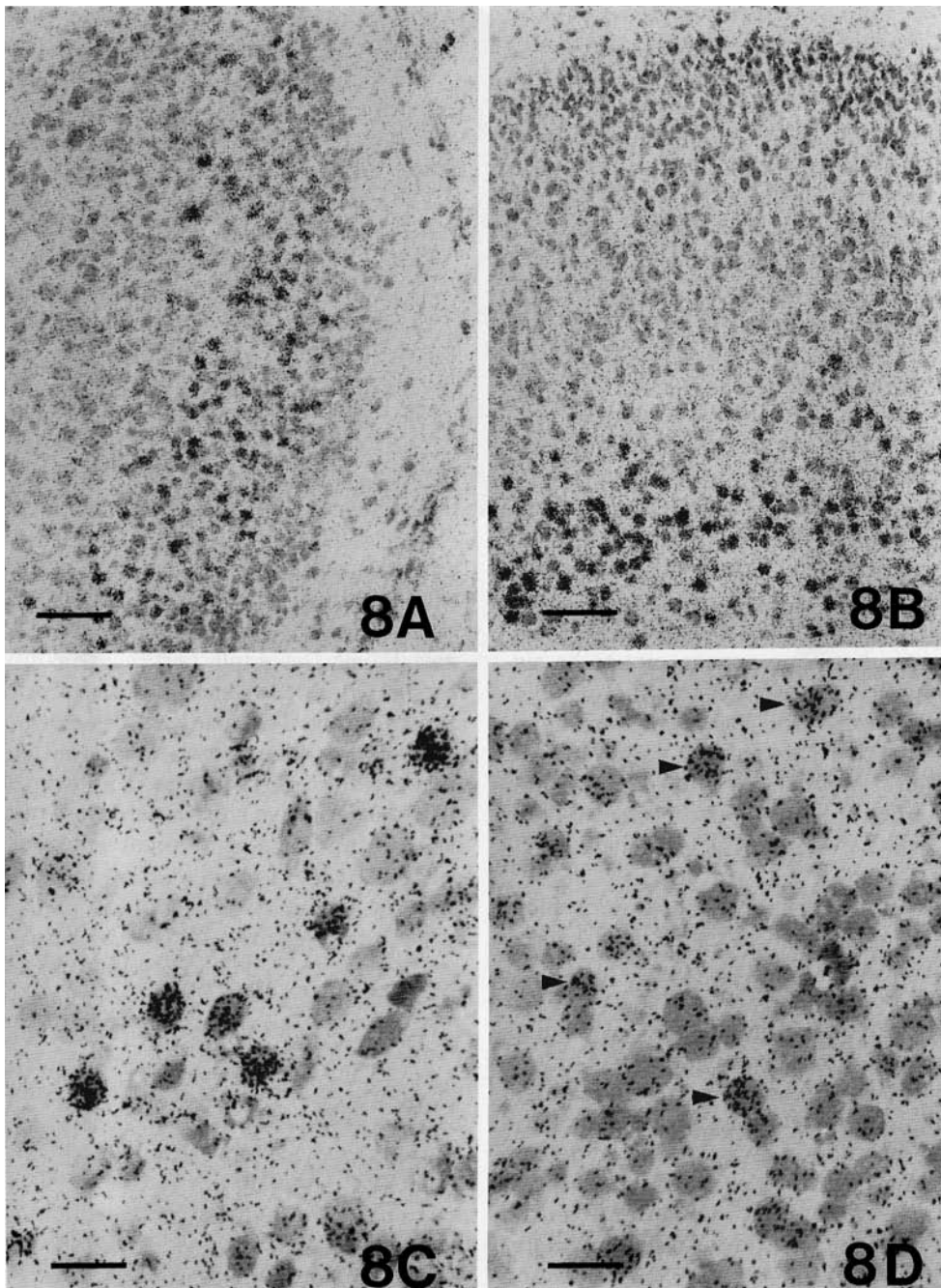
regions, whereas labeling in laminae II and III was similar to that in the 8-day postnate. On day 18, an attenuation in labeled cell number and topography was seen throughout the cortex. By day 25, a small cluster of estrogen target cells remained in laminae II and III of the dorsal paracingulate and ventral suprarhinal transitional cortex, with a few labeled cells scattered among the remaining regions. The developmental changes in the dorsal paracingulate (figure 43.5) and ventral suprarhinal cortex (figures 43.6 and 43.7) were compared at the level of the preoptic area. Additional regions that displayed a diminished number of labeled cells with brain maturation, such as the cingulate (figure 43.8A) and occipital (figure 43.8B) cortex, have also been presented. While a sexual dimorphism in receptor cell number or distribution was not apparent, subtle differences in the labeling intensity between the sexes and the left vs. right hemispheres cannot be excluded.

Estrogen target cells viewed at high magnification had a concentration of radioactivity in the cell nucleus (figure 43.8, C and D). Administration of unlabeled

estradiol 1 h before radiolabeled estradiol reduced the nuclear concentration of radioactivity in all labeled regions, including the deep frontal cortex (figure 43.9).

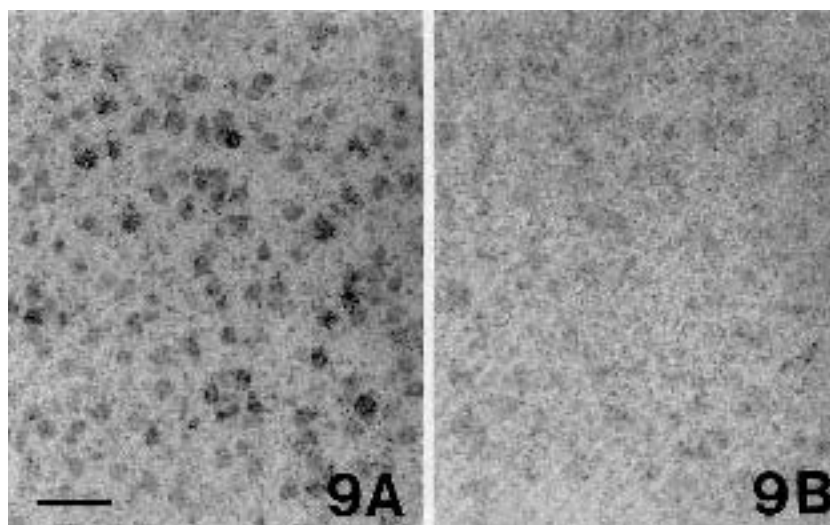
### Discussion

The present autoradiographic investigation with [ $^{125}$ I]MIE<sub>2</sub> has demonstrated the presence of estrogen target cells in postnatal mouse cerebral cortex and shown that extensive reorganization and attenuation of this system occur with brain maturation. Estrogen target cells with weak nuclear uptake and retention of radioactivity were found throughout the deep cortex at birth, increased in deep layers until day 2, and then diminished to a few cells by postnatal day 25. On day 2, additional labeled cells appeared in the intermediate and superficial layers of the dorsal cingulate/paracingulate and ventral suprarhinal cortex. These target cells increased in number and regional distribution until day 12, and then decreased in number between days 12 and 25. While the regional and laminar distribution of estrogen target cells in the 25-day



**Figure 43.8**

Autoradiograms of cortical cells with nuclear uptake and retention of radioactivity after sc injection of [ $^{125}$ I]MIE<sub>2</sub>. Labeled cells were observed in the superficial laminae of the 2-day retrosplenial granulos cortex (A) and deep layers of the lateral occipital cortex (B). Target cells in the deep cortex at the preoptic level were strongly labeled in the 2-day postnate (C), while cells in the 0-day cortex appear to be weakly labeled (*arrows*, D). Exposure was for 28 days. Magnification: A,  $\times 200$ ; B,  $\times 190$ ; C,  $\times 640$ ; D,  $\times 410$ . Bar: A and B, 50  $\mu$ m; C, 15  $\mu$ m; D, 25  $\mu$ m.



**Figure 43.9**

Autoradiograms of the deep frontal cortex of the 2-day postnate after sc injection of [ $^{125}$ I]MIE $_2$ . The nuclear concentration of radioactivity observed in the deep laminae (A) was reduced after competition with unlabeled 17 $\beta$ -estradiol (B). Exposure was for 28 days. Magnification,  $\times 200$ . Bar = 50  $\mu$ m.

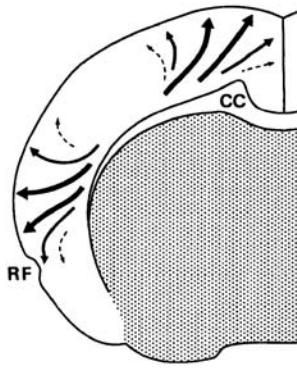
postnatal cortex, described herein, was similar to the pattern in the adult female mouse (27), numerical differences suggest that a further reduction in target cell number occurs between postweaning and adulthood. Injection of unlabeled 17 $\beta$ -estradiol 1 h before radiolabeled estradiol reduced the nuclear concentration of radioactivity and demonstrated the specificity of [ $^{125}$ I]MIE $_2$  for the estrogen receptor.

During autoradiographic investigation of the mouse frontal/cingulate cortex, Gerlach et al. (15) found that estrogen receptor cells first appear on fetal day 18, and during the first 9 days of postnatal life either increase in number (cingulate cortex) or remain unchanged (frontal cortex). The results of the present experiments also demonstrate that a dramatic increase occurs in the deep and superficial cingulate cortex between birth and postnatal day 8, but in contrast to Gerlach et al., we detected an increase in the frontal cortex as well as in many other regions throughout the 8-day postnatal cortex.

In the present study we found a decline in the number of target cells from day 8 to day 25. Comparison of the vast labeling in the deep layers of the 2-day postnatal rat cortex (13) with the diffuse labeling in the adult (14) suggests that a similar reorganization and attenuation of receptor cells occurs in the rat. This concept is in agreement with and supported by biochemical evidence which showed that the number of estrogen receptors in the rat cortex declined after the first week of postnatal life (19). An autoradiographic investigation by Presl et al. (28) was unable to detect any change in the distribution of estrogen receptor cells in the rat cor-

tex between postnatal days 5 and 50, although these results have not been confirmed or refuted.

Biochemical studies of the mouse cortex (20) also revealed that the number of estrogen receptors rapidly increased and peaked during the first postnatal week and then declined with maturation until postweaning. These results were similar to those reported in the present study, although several notable differences during the first week of life were observed. Friedman et al. (20) found that the number of receptors increased steadily from postnatal day 5 to day 9, while our results suggest that the absolute number of cortical receptor cells was highest on day 2 and then gradually declined with postnatal development. One explanation for this discrepancy is that the change in receptor number does not reflect the number of target cells present in the early postnatal cortex. Since the present study suggests that the intensity of nuclear labeling increases in target cells from birth to day 8, comparing the number of receptors vs. cells may not accurately depict developmental changes in cell number during early postnatal life. When the number of estrogen receptors present in cells appears to be more constant, after the first postnatal week, changes in receptor number were in agreement with changes in cortical target cell number. An alternative explanation for the difference between the biochemical and the present autoradiographic results is a partial competition by maternal estrogens with the radiolabeled ligand. Competition by endogenous hormone appears not to be significant, since cells with a dense concentration of radioactivity in the nucleus were seen in the preoptic hypothalamus and repro-



**Figure 43.10**

Schematic representation of estrogen receptor cells between birth and postweaning, depicted as vectors (*arrows*), and their hypothesized migration through the cerebral cortex. Labeled cells may migrate in the deep cortex toward the dorsal paracingulate and ventral suprarhinal transition cortex, leaving a sparse region of labeling in the isocortex between these labeled periallocortical regions. CC, Corpus callosum; RF, rhinal fissure.

ductive tract of the same animals (our unpublished observations). In addition, previous autoradiographic studies of the fetal and perinatal brain were able to detect cells with strong nuclear labeling (15–18).

Although the mechanism underlying the developmental redistribution of estrogen receptor cells remains unclear, the results of the present investigation suggest that target cells may be migrating from the deep cortex to the superficial layers of the cingulate/paracingulate and suprarhinal regions during postnatal development. This migration appears to begin on day 2, when target cells begin to accumulate in the deep, intermediate and superficial layers of the dorsal cingulate/paracingulate and ventral suprarhinal regions. The migration of target cells through lamina VI toward the dorsal and ventral regions might explain the increase in the 8-day cingulate/paracingulate and suprarhinal cortex and the reduction between these regions. As cells migrate toward laminae II and III of the cingulate/paracingulate and suprarhinal regions, one would expect to see an increase in laminae II and III, with a subsequent decrease in lamina V and VI. This pattern of labeled cells was observed in the 12-day postnate and was maintained until day 25, when a slight attenuation in cell number was observed. Taken together these observations suggest that the changes in the topography of estrogen target cells during postnatal life are the result of cell migration from deep layers to the dorsal and ventral superficial laminae of the cortex (figure 43.10).

In the rat, neuroblast migration occurs from midgestation until the end of the first postnatal week (29). At birth, most of the neuroblasts destined for laminae II and III of the adult cortex are confined to the deep cor-

tex, while the superficial layer is composed of cells that will form laminae V and VI (29).

During the first postnatal week the deep neuroblasts migrate through the superficial layer of cells and establish the definitive superficial layers of the mature cortex (29). Comparison of this pattern of migration in the rat with the redistribution of estrogen target cells we observed in the mouse further suggests that labeled cells migrate from deep to superficial laminae during early postnatal life.

An alternative explanation for the topographical change in estrogen receptor cells during postnatal development is a differential maturation of the cortex. Since the deep cortex is established before the superficial layers, according to the “inside outside” model for cortical development (29), deep neuroblasts would be expected to send out neurites and establish synapses before superficial cells. If estradiol was important for the maturation of certain cells during this phase of development, one would expect to see extensive labeling in the deep cortex, followed by labeling in the superficial layers. Once the estrogen-sensitive period had ended, and cell differentiation occurred, certain target cells might cease expression of the estrogen receptor.

Although the significance of these topographical changes remains unknown, recent evidence suggests that estradiol may synergize with insulin-like growth factor(s) to promote neurite outgrowth of estrogen receptor-containing cells in the postnatal cerebral cortex (30). These results in conjunction with the present findings suggest that estrogen may be essential for the connectivity of certain target cells during a restricted period of postnatal life.

Previous investigators found that estradiol administered during early postnatal life accelerated the appearance of an adult-like seizure pattern in the rat (31), possibly by accelerating the rate of myelination in sensory-motor cortex (32). The presence of estrogen target cells in the motor-sensory cortex of the mouse, described in the present paper, suggests that this may be a receptor-mediated event.

Additional studies found that the quantity of estrogen present during the critical period of brain development has a differential effect on cortex morphology and function. When the thicknesses of the male and female rat cortex were compared, researchers found that the right male hemisphere was thicker than the left (33), and that gonadectomy at birth altered this development (34). Male mice also exhibit lower levels of open-field activity compared to females. If a female rat was ovariectomized during postnatal life and tested as an adult, the open-field score was lower than that in females ovariectomized and tested during adulthood (35). When a low dose of estradiol was administered to

gonadectomized male and female rats, an increase in ambulation was observed during adulthood compared to that in oil-treated controls. Conversely, a high dose of estradiol administered before postnatal day 10 reduced female open-field activity (36).

The presence and activity of aromatase, an enzyme that converts androgens to estrogens (11, 12), is thought to establish a sexual dimorphism in estrogen levels during cortical development (37, 38). Since the present investigation was unable to detect a male/female difference in estrogen receptor number or distribution, the aromatization of male androgens within the developing cerebral cortex may result in a sexual dimorphism in estrogen available to target cells. Subtle differences in the number of receptors per target cell, a possibility that cannot be excluded, may also account for morphological and functional differences in the adult cortex.

In this study we have demonstrated the presence of estrogen receptor cells in the postnatal cortex and documented developmental changes in their topography and number. Based on these observations estrogen appears to be important for the development of certain cortical regions, and a sexual dimorphism in the estrogen available to these cells could account for a sexual dimorphism in cortical function.

### Acknowledgments

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Males undergo two processes during development that affect their adult behavior. Masculinization refers to the underlying neural circuitry and behavioral patterns that are exhibited to a greater degree by males than females. For example, males of many species display a set of sex specific courtship and copulatory behaviors. In addition, a separate process, defeminization, reduces the likelihood that males will display female-typical behaviors in adulthood, such as display of the receptive mating posture, lordosis. Many sexual dimorphisms in brain and behavior are caused by developmental sex differences in steroid hormones that act on nuclear receptors (1). Specifically, neonatal testes produce testosterone for a finite period beginning at the end of gestation until shortly after birth (2). Testosterone is aromatized neurally to estradiol (E2) and binds to two known estrogen receptors (ER $\alpha$  and ER $\beta$ ) (3). Depriving males of their testes, or steroids produced by the testes, during this developmental period results in demasculinization and feminization (4–6).

The mechanism by which estradiol affects both masculinization and defeminization is unknown. Here, we test the hypothesis that these processes are regulated by different ERs. We hypothesize that ER $\beta$  has a specialized function in the development of a sexually differentiated behavior and is essential for defeminization. This hypothesis is supported by the report of sex differences in ER $\beta$  in neonatal mice; during late gestation and the first 2 weeks after birth, males have significantly more ER $\beta$  mRNA than females in the medial basal hypothalamus including the medial preoptic area (POA) (7).

To test our hypothesis, we used male ER $\beta$  knockout (ER $\beta$ KO) mice along with their WT littermates (8). Adult male and female ER $\beta$ KO mice can perform sex-typical sexual behaviors (9–11), and males are fertile (8). We predicted that if ER $\beta$  is exclusively involved in defeminization of the male brain, male ER $\beta$ KO mice would fail to undergo complete defeminization during development and, as a consequence, when tested in adulthood they would display more female-type receptivity than WT littermates. However, the lack of ER $\beta$  should have no impact on the masculinization process

and, thus, male ER $\beta$ KO mice should display equivalent masculine sexual behavior as compared with WT males. We assessed a second sexually dimorphic social behavior, olfactory preferences. Male mice show a strong preference for female-soiled bedding over male-soiled bedding (12, 13). We hypothesized that WT and ER $\beta$ KO males would both preferentially investigate female-soiled bedding vs. male-soiled or clean bedding.

## Methods

### Animals

The mice were generated by mating heterozygous carriers of the disrupted ER $\beta$  gene (8), and the offspring genotype (ER $\beta$ KO or WT) was determined by PCR amplification of tail DNA, as described in ref. 14. The individuals were of a mixed 129/SvJ and C57BL/6J background, backcrossed into the C57BL/6J strain for five generations (making them, on average, 97% similar to the inbred C57BL/6J). All males were weaned between 18 and 20 days of age and group-housed until they were between 50 and 60 days old. Males were individually housed either after castration (experiments 1 and 3) or beginning 3 days before testing (experiment 2) for the entire duration of testing, with food (Purina mouse chow no. 5001) and water available ad libitum. All individuals were kept on a 12-h light:dark cycle with lights off at 1200 hours.

### Experiment 1

**Female Sexual Behavior** Thirty-eight adult male mice (70–100 days of age) were used in this study, 16 WT and 22 ER $\beta$ KO. Mice were gonadectomized under general anesthesia (100 mg/kg ketamine and 10 mg/kg xylazine injected i.p.). Seven to 10 days after surgery, males were injected s.c. with estradiol benzoate (EB) (0.5  $\mu$ g dissolved in 0.05 ml of sesame oil). Two days later, progesterone (400  $\mu$ g in 0.03 ml of sesame oil) was administered s.c. 3–4 h before the onset of a sexual behavior test. Animals were tested seven times with 4–5 days between trials.

**Receptivity Tests** All sexual behavior tests were conducted starting 2 h after lights off, under red-light illumination. All testing was conducted in 18 × 38-cm Plexiglas test chambers that were placed on a mirror stand to allow for ventral viewing. Sexually experienced C57BL/6J and DBA/2J males were used as testing partners. Each stud male was habituated for 1 h in the test chamber before the introduction of the hormone-primed experimental male. All tests were terminated after the experimental males received 20 mounts from the stud male (defined as both forepaws on the hind region) or after 15 min, whichever occurred first. We defined lordosis by using the following stipulations: all four paws were grounded, the hind region was elevated off the floor of the test chamber, and the back was slightly arched. The lordosis quotient (LQ; lordosis number/number of mounts) was scored for each subject.

## Experiment 2

**Male Sexual Behavior** Twenty-five adult (60–120 days of age), gonad-intact males, 11 WT and 14 ER $\beta$ KO, were used. Males received social exposure (15) for 3 consecutive days before sexual behavior testing commenced. Each male was then tested three times for male sexual behavior with an interval of 2–3 days between each consecutive test. However, once the male performed an ejaculation during a test, his testing sequence ended. One week after the final test, animals underwent olfactory preference testing (described below).

**Stimulus Females** Stimulus females (C57BL/6J) were ovariectomized after 40 days of age and implanted with a Silastic implant (1.96-mm i.d. × 3.18-mm o.d.) filled with EB (50  $\mu$ g dissolved in 30  $\mu$ l of sesame oil). Three to 5 h before the tests, the females were injected s.c. with progesterone (1 mg dissolved in 60  $\mu$ l of sesame oil), and they were prescreened with a sexually experienced male to ensure receptivity.

**Male Sexual Behavior Tests** Sexual behavior tests were conducted in the same test boxes and at the same time of day as described in experiment 1. Each subject was habituated to the testing box for 30 min before a hormone-primed receptive female stimulus animal was introduced. Tests lasted for 30 min if no sexual behavior was displayed. If a mounting episode was displayed within the first 30 min, the test was extended for 60 additional min, or until the male performed an ejaculation, whichever occurred first. If a subject displayed an ejaculation, the test was terminated and the male was not tested again. The variables recorded included latencies to mount, thrust, intromit, and ejaculate and the

number of mounting bouts and thrusts per mounting bout. Latency scores were cumulative over each test period. Frequency data were analyzed from the test during which the maximum amount of sexual behavior occurred.

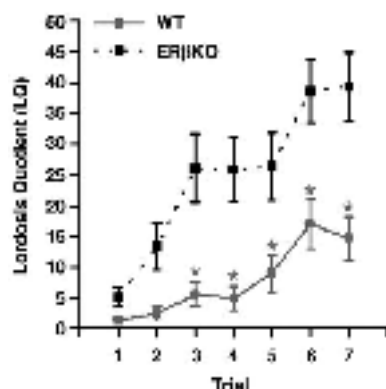
## Experiment 3

**Olfactory Preference Tests** Males tested for sexual behaviors in experiment 2 were also tested for olfactory preferences. The detailed methods of our olfactory preference test are published in ref. 16. Tests were conducted between 3 and 8 h after room lights went out under red-light illumination. In brief, the experimental setup consisted of three small plastic containers filled with bedding located equidistant from each other inside the standard Plexiglas test box. For the habituation period, all containers contained clean bedding. Next, mice were removed from the test box, and three different containers containing different types of bedding (i.e., male-soiled, female-soiled, and clean) were placed in the box. Tests lasted for 10 min, and the time spent actively sniffing each container (the male's nose had to be in direct contact with the bedding) was recorded. The observer was blind to the content of each container.

**Stimulus Bedding** Twenty-four hours before bedding was collected, group-housed (four per cage) gonadectomized females that had s.c. EB-filled Silastic implants (1.96-mm i.d. × 3.18-mm o.d.; 50  $\mu$ g of EB dissolved in 30  $\mu$ l of sesame oil) were placed into a cage with clean bedding. The females received a progesterone injection 6 h before bedding collection. Soiled bedding from adult males (C57BL/6J) individually housed on clean bedding for 24 h was obtained from four cages.

## Data Analysis

Female sex behavior data were analyzed by using a repeated-measures ANOVA with genotype and trial as the factors. Comparisons among trials and genotypes were analyzed by using Bonferroni multiple comparison tests, which correct for the number of comparisons made. In experiment 2, the frequencies of males of each genotype performing various behaviors were compared with Fisher exact tests. We analyzed male sexual behaviors, only including data from males that displayed the behaviors, with one-way ANOVA. A repeated-measures ANOVA with bedding as the repeated measure and genotype as the factor was used to analyze the data from the olfactory preference tests. Bonferroni multiple comparison tests were used for the post hoc analysis. In all cases, significance was reported at  $P < 0.05$  or less.



**Figure 44.1**

Mean ( $\pm$ SEM) LQ for castrated WT ( $n = 16$ ) and ER $\beta$ KO ( $n = 22$ ) male mice. Males received s.c. injections of EB and progesterone 48 and 3 h, respectively, before each behavior test. Tests were conducted at 4- to 5-day intervals. \*, WT males displayed significantly lower levels of lordosis as compared with ER $\beta$ KO males on each trial ( $P < 0.05$ ).

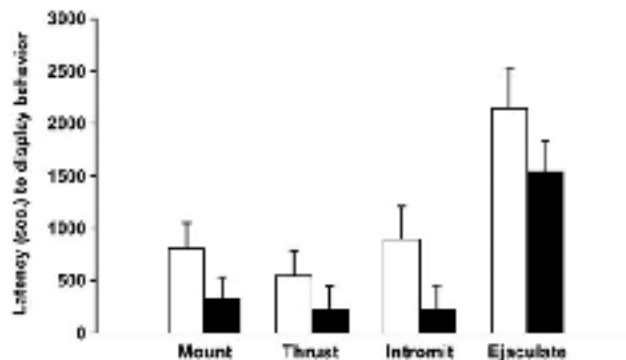
## Results

### ER $\beta$ KO Males Display Enhanced Female Sex Behavior

Castrated ER $\beta$ KO males treated with EB and progesterone displayed female-like receptivity, defined as holding the lordosis posture and allowing males to mount and thrust. A one-way repeated-measures ANOVA revealed effects of genotype [ $F_{(1,265)} = 18.37$ ;  $P < 0.001$ ] and trial [ $F_{(6,265)} = 13.14$ ;  $P < 0.01$ ]. The interaction between genotype and trial approached significance [ $F_{(6,265)} = 2.10$ ;  $P = 0.054$ ]. Overall, ER $\beta$ KO males displayed significantly higher LQs as compared with WT littermates (figure 44.1), and as with female mice, the LQ scores of ER $\beta$ KO males increased over trials; LQ on trials 6 and 7 were significantly greater than on trials 1–4 ( $P < 0.05$ ). Furthermore, post hoc analysis with Bonferroni multiple comparison tests revealed that ER $\beta$ KO males exhibited a higher LQ compared with WT males on every trial ( $P < 0.05$ ) except for trials 1 and 2.

### Male Sexual Behavior Is Unaffected by Lack of Functional ER $\beta$

The majority of the males tested, 10 (of 14) ER $\beta$ KO and 8 (of 11) WT mice exhibited sexual behavior in at least one test. These proportions were not significantly different ( $P = 0.94$ ). In total, eight ER $\beta$ KO and five WT individuals achieved complete sequences of sexual behavior, including ejaculation, during the testing series. There were no differences between the genotypes in the latencies to mount [ $F_{(1,20)} = 2.29$ ], thrust [ $F_{(1,19)} = 0.88$ ], intromit [ $F_{(1,16)} = 3.02$ ], or ejaculate [ $F_{(1,11)} = 1.64$ ] (figure 44.2). The number of intromissions displayed before ejaculation, the latency between the first intromission and ejaculation, and the



**Figure 44.2**

Mean ( $\pm$ SEM) latency to first mount, thrust, intromission, and ejaculation for testes-intact WT and ER $\beta$ KO mice in male sexual behavior tests. Latency to first mount was calculated from the onset of the testing period; all other latencies were calculated from the onset of the first mount displayed. No significant differences were found between WT and ER $\beta$ KO males. White histograms are data from WT, and black histograms are data from ER $\beta$ KO males.

average number of thrusts with intromissions per mounting bout were not significantly different between the groups [ $F_{(1,11)} = 0.18, 0.71$  and  $F_{(1,16)} = 0.46$ , respectively] (table 44.1). When the total number of mounting episodes were normalized according to the amount of time that each subject had been tested, the differences remained nonsignificant [ $F_{(1,20)} = 1.80$ ] (table 44.1).

### Olfactory Preferences Are Equivalent Between WT and ER $\beta$ KO Males

Olfactory preference tests conducted in sexually experienced, testes-intact mice revealed a significant effect of bedding type [ $F_{(2,69)} = 35.71$ ;  $P < 0.0001$ ] but not of genotype, and there was no significant interaction between the two factors. The post hoc analysis showed that all males preferred to spend significantly more time sniffing soiled bedding compared with the clean bedding used as a control, and both genotypes showed a significant preference for bedding previously soiled by estrous females over bedding soiled by males ( $P < 0.05$ ) (figure 44.3).

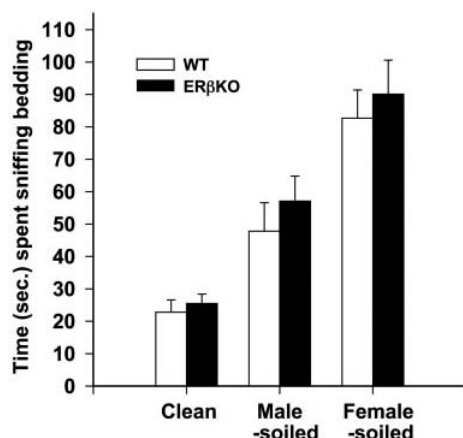
## Discussion

Our major finding is that male mice lacking functional ER $\beta$  are incompletely defeminized. When treated with the appropriate hormonal priming, male ER $\beta$ KO mice display significantly more female-like sexual receptivity than WT littermates. Yet, lack of functional ER $\beta$  does not impair normal expression of adult masculine sexual behavior or olfactory preference in testes-intact males (table 44.2). When standard tests for male sexual behavior were performed, WT and ER $\beta$ KO males showed equivalent latencies to perform the various

**Table 44.1**Values (mean  $\pm$  SEM) for variables recorded on the tests for masculine behavior

Variable	WT	ER $\beta$ KO
Number of thrusts with intromissions per mounting episode	22.92 $\pm$ 8.25 (6)	29.76 $\pm$ 5.83 (12)
Total number of thrusts with intromissions preceding ejaculation	367.38 $\pm$ 59.05 (5)	326.80 $\pm$ 74.69 (8)
Total number of mounting episodes	29.92 $\pm$ 6.32 (9)	19.56 $\pm$ 7.60 (13)
Number of mounting episodes preceding ejaculation	28.50 $\pm$ 6.57 (5)	32.60 $\pm$ 8.31 (8)

The numbers of animals in each group is given in parentheses. No significant differences were registered between the groups.

**Figure 44.3**

Mean ( $\pm$ SEM) time in seconds spent chemoinvestigating one of three bedding choices (clean, male-soiled, and female-soiled). Males were testes-intact and sexually experienced (10 WT and 13 ER $\beta$ KO). Although no genotype effect was found, males did spend significantly different amounts of time sniffing the three bedding type: the least time was spent investigating clean bedding, then male-soiled bedding, and, finally, the most time was spent sniffing female-soiled bedding ( $P < 0.05$ ).

**Table 44.2**Summary of behavioral differences between WT and ER $\beta$ KO male mice

Behavior	WT	ER $\beta$ KO
Masculine sexual	+	+
Masculine olfactory	+	+
Feminine sexual	–	+

components of copulatory behavior, and the frequencies with which they displayed each behavior were also similar. Both WT and ER $\beta$ KO males have a distinct preference for female-soiled bedding over male-soiled or clean bedding. Olfactory preferences may be an assay of sexual interest and/or motivation (12), and, thus, our findings indicate that ER $\beta$ KO mice have normal interest in female olfactory cues. In summary, we find no evidence that masculinization is deficient in ER $\beta$ KO males; however, we propose that the defeminization process is incomplete in these animals.

The mechanisms by which ER $\beta$  affects defeminization are unknown. However, the idea that masculiniza-

tion and defeminization may be uncoupled in males is not new (17). Castrated adult male ferrets can display female-like receptive behavior upon receipt of the appropriate priming hormones, but they also display typical male sexual behavior when tested with normal circulating levels of testosterone or with their testes intact (18, 19). Thus, male ferrets are masculinized but not defeminized, similar to the ER $\beta$ KO mouse. Lesions of the sexually dimorphic nucleus (SDN) in the male ferret POA/AH block female-like preferences for stud males but have little impact on male sexual behavior (20), suggesting that this region is involved in defeminization. This steroid-sensitive nucleus in the ferret is anatomically similar to the SDN of the POA found in rats and other mammals (20–22). However, mice have fewer documented sexual dimorphisms in brain than rats, and C57BL/6J mice do not possess a SDN (23, 24). Yet, in both neonatal and adult mouse brains, sex differences in ER $\beta$  have been demonstrated (7, 25). Specifically, between embryonic day 17 and postnatal day 15, males had more ER $\beta$  mRNA than did females (7), and in adult mice, castrated C57BL/6J males have more ER $\beta$  immunoreactive cells throughout the medial POA than do adult ovariectomized females (25). We suspect that this sex difference in ER $\beta$  is related to defeminization of the male brain. The influence of ER $\beta$  on defeminization may involve the progesterin receptor (PR). In adult male C57BL/6J mice, but not female C57BL/6J mice, ER $\beta$  is involved in estradiol-regulation of PRs. Specifically, maximal PR induction in male mice requires at least one functional copy of ER $\beta$  (25). Because PR is also essential for the expression of female receptivity in rodents (26), we speculate that normal defeminization involves PR expression, which may be regulated in males by ER $\beta$ .

In adulthood, ER $\beta$ KO males and females can display normal sex-specific copulatory behaviors (9–11). In fact, ER $\beta$ KO females display more regular estrous cycles and enhanced receptivity as compared with WT littermates (9, 11, ¶). After equivalent treatment with estradiol, ER $\beta$ KO females tend to have more PR-immunoreactive neurons in the ventromedial nucleus of the hypothalamus than WT females (14). Thus, a lack of ER $\beta$  could enhance feminization in females. Female mice display varying degrees of feminine be-

havior as adults, and this may be attributed to differences in exposure to steroid hormones in utero (27). Depending on uterine position, some female embryos can be exposed to androgens from their male siblings. This testosterone may be aromatized to E2 in the brain and, via ER $\beta$ , may have a mild defeminizing effect in females. In males, sexual behavior in adult ER $\beta$ KO mice is equivalent to WT males, but transient developmental differences in both aggressive and sexual behaviors during puberty have been observed in male ER $\beta$ KO mice (10, 28). It is possible that these behavioral differences are based on the same neural circuitry that produces the propensity for male ER $\beta$ KO mice to display enhanced lordosis behavior.

Data from KO mice suggest that, in females, ER $\alpha$  is essential for normal sexual behavior and fertility (29). Females lacking ER $\alpha$  or both ER $\alpha$  and ER $\beta$  are infertile (30) and fail to display receptivity after hormone priming (9, 31, 32). Because the lack of functional ER $\alpha$  has such pronounced effects, ER $\alpha$ KO mice cannot be used to explore the role of ER $\beta$  in female reproduction. However, data from female rats support the possibility that ER $\beta$  may affect female reproduction, at least in some brain areas. Treatment of female rat pups for the first 12 days of life with estradiol, an ER $\alpha$ , or an ER $\beta$ -selective agonist decreases the number of neurons in the sexually dimorphic anteroventral periventricular region (AVPV) (33). In that area, all estrogen treatments reduced the number of neurons per unit area, thus making the region more male-like than female-like (i.e., defeminized). Rat brains display a sex difference in ER $\beta$  message in AVPV starting on postnatal day 7 and continuing into adulthood (34); females have more ER $\beta$ -mRNA-containing cells than do males, and this difference can be reversed by early treatment of females with estrogen or castration of male pups. Thus, the rat data suggest that ER $\beta$  may modulate cell numbers in the AVPV and, by inference, interfere with reproduction. Presently, we are treating neonatal mice with ER $\alpha$  and ER $\alpha$ -selective agonists and examining adult sexual behavior. Our preliminary data show a defeminizing effect of the ER $\beta$  agonist, but not an effect of the ER $\alpha$  agonist, on adult female sexual behavior (A.E.K. and E.F.R., unpublished data). Clearly, differences exist between mice and rats, and ER $\beta$  may not have precisely the same role in each species. However, the collective data from both species suggest that ER $\beta$  activation during development can influence defeminization in brain.

Another line of evidence that supports our hypothesis that ER $\beta$  is involved in defeminization comes from studies investigating the effects of early exposure to phytoestrogens on adult behavior. Phytoestrogens bind preferentially to ER $\beta$  (35) and generally have anti-estrogenic actions in female rats. Female rat pups

injected with the phytoestrogen genistein daily for the first 5 days of life showed a reduction in lordosis behavior in adulthood (36). Female sexual behavior is suppressed in aromatase enzyme KO mice (37). However, this finding was only replicated when females were raised on a phytoestrogen-rich diet (38). Again, the mechanism may be that phytoestrogens activate ER $\beta$  and, thus, defeminize receptive behavior.

The complementary process to defeminization is masculinization. Based on data presented here and on unpublished data in which female ER $\beta$ KO mice treated with T displayed normal levels of mounting and thrusting behavior when tested with receptive females (A.E.K. and E.F.R., unpublished data), we suggest that ER $\beta$  is not required for masculinization. It is tempting to speculate that ER $\alpha$  instead is responsible for masculinization. Mice lacking functional ER $\alpha$  show a number of behavioral impairments including the failure of both males and females to exhibit masculine sexual behavior when they are tested with the appropriate hormones in adulthood (15, 39, 40). Male ER $\alpha$ KO mice show low levels of masculine sexual behavior (40, 41) and have no preferences for awake females versus males, anesthetized females versus males, or female versus male-soiled bedding (42). Although these data are consistent with the hypothesis that masculinization requires ER $\alpha$ , it is not yet possible to determine whether the actions of ER $\alpha$  are essential during development and/or in adulthood. The test of this hypothesis awaits development of mouse models with temporal control of ER $\alpha$  expression.

In summary, we hypothesize that ER $\beta$  plays an essential role in sexual differentiation of brain and behavior. Whether defeminization also requires ER $\alpha$  is not clear, but interactions between the two receptors within several brain nuclei, including those involved in sexual behavior, have been well documented (14, 43, 44). Moreover, the pattern of response to estradiol in the ventromedial nucleus of the hypothalamus and POA of male ER $\beta$ KO mice is feminized for both ER $\alpha$  and PR regulation (14), and it is likely that both of these receptors are involved in expression of receptivity. Past work suggesting neural (14, 45, 46) and behavioral (10, 47, 48) deficiencies in ER $\beta$ KO mice need to be evaluated with this hypothesis in mind. This evaluation could lead to new applications of estrogen-based treatments for sexually dimorphic neurological diseases.

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Two mammalian estrogen receptor (ER) genes are now known, encoding, respectively, ER- $\alpha$  (~67 kDa) (White et al., 1987), which mediates many of the known transcriptional actions of estrogen in the brain, and the more recently cloned ER- $\beta$  (Kuiper et al., 1996) (60 kDa in mouse ovary) (Fitzpatrick et al., 1999), whose neural role is less well defined. A third, more distantly related member of the ER family, ER- $\gamma$ , was recently cloned in teleosts only (Hawkins et al., 2000). ER- $\alpha$  and ER- $\beta$  appear to be complementary but not redundant. Under steady-state conditions they are predominantly intranuclear and differ with respect to the homology of their functional domains, binding affinities, and ligand specificities (Kuiper et al., 1997). ER- $\alpha$  and ER- $\beta$  act as ligand-inducible transcriptional enhancers (Landers and Spelsberg, 1992; Beato and Klug, 2000), binding to cognate estrogen response elements (EREs) in DNA to regulate gene expression. Their spatiotemporal expression and distribution differ with developmental stage. Thus, although neocortical ER- $\beta$  is present throughout life (Shughrue et al., 1997), neocortical ER- $\alpha$  expression is developmentally regulated and normally expressed at high levels only during neocortical differentiation (Shughrue et al., 1990, 1997), suggesting a more restricted developmental role.

Some responses to estradiol cannot be attributed to ER- $\alpha$  or ER- $\beta$  (Singh et al., 1999, 2000), such as the ability of estrogen to regulate non-ERE-containing genes (Sukovich et al., 1994) and the very rapid (seconds to minutes) effects of estrogen (Kelly et al., 1978; Chiaia et al., 1983; Garcia-Segura et al., 1987; Migliaccio et al., 1993). Whereas such rapid responses appear inconsistent with direct transcriptional modulation via intranuclear receptors, they could be explained by the presence of plasma membrane-associated ERs that may be coupled to signal transduction pathways, typically associated with rapid activation by growth factors.

Neurotrophin activation of the mitogen-activated protein kinase (MAPK) cascade is mediated by cognate transmembrane receptors associated with caveolar-like microdomains (CLMs) of neuronal

plasma membranes, the neuronal homologs of caveolae found in most cell types other than neurons (Huang et al., 1999). Caveolae/CLMs form important signaling modules that compartmentalize, modulate, and integrate growth factor-induced signaling events at the cell surface (Anderson, 1998; Okamoto et al., 1998). Like the neurotrophins, estrogen is an important neural trophic factor throughout life, with influences on neuronal differentiation (Toran-Allerand, 1976, 1980), survival (Green and Simpkins, 2000; Garcia-Segura et al., 2001), and plasticity (Matsumoto and Arai, 1981). 17 $\beta$ -estradiol activates many signaling kinases, including protein kinase C (PKC) (G. Sétáló, Jr. and C. D. Toran-Allerand, unpublished observations), c-src (Nethrapalli et al., 2001), and members of the MAPK cascade (Watters et al., 1997; Singh et al., 1999, 2000). Rapid and sustained activation of cytoplasmic ERK1/2 is followed by nuclear translocation of phosphorylated ERK (Sétáló et al., 2001). We have proposed (Singh et al., 1999, 2000) that the ER mediating estrogen-induced activation of ERK1/2 in the developing brain is neither ER- $\alpha$  nor ER- $\beta$  and have hypothesized that, like neurotrophin receptors, this ER might be associated with CLMs (Toran-Allerand, 2000). Here we provide evidence for the existence of a novel and unique, plasma membrane-associated (CLM-associated) putative ER that we have designated "ER-X" (Toran-Allerand, 2000).

## Materials and Methods

All animal experiments were conducted in a humane manner, and animals were maintained according to protocols approved by the Institutional Animal Care and Use Committee at Columbia University. To identify and characterize ER-X, we analyzed, by immunoprecipitation, Western blotting and both light and electron microscopy, cell lysates, detergent-free, highly purified CLM preparations (Smart et al., 1995), plasma membranes, postnuclear supernatants (PNS), and tissue sections obtained from postnatal day 7 (P7) and adult wild-type and ER- $\alpha$  gene-disrupted (ERKO) neocortex and uterus.

### Mice

Wild-type and ERKO mice were obtained from our breeding colony from matings of C57BL/6J  $\times$  129 mice heterozygous (+/–) for the ER- $\alpha$  gene disruption (Lubahn et al., 1993) and identified by genotyping (Singh et al., 2000) as either wild-type (+/+) or homozygous (–/–) for the disruption.

### Genotyping

Tail snips were obtained from P3–P4 pups and used for genotyping, as previously described (Singh et al., 2000). Briefly, tissues were digested with Proteinase K at 56°C for 90 min, followed by a 99°C incubation for 10 min. The samples were then vortexed vigorously, and insoluble material was pelleted in a microfuge. Supernatants were used in a PCR reaction that used one primer pair [primer 1: 5'-CGG TCT ACG GCC AGT CGG GCA TC-3'; primer 2: 5'-GTA GAA GGC GGG AGG GCC GGT GTC-3'] for the ER- $\alpha$  gene product (product size, 239 bp), and one primer pair [primer 2 from above with NEO Primer: 5'-GCT GAC CGC TTC CTC GTG CTT TAC-3'] for the neomycin insert-containing gene product (product size, 790 bp). The PCR program was performed as follows: 1 cycle at 94°C for 3 min, 30 cycles of 94°C for 45 sec, 62°C for 1 min, and 72°C for 1 min 40 sec, followed by a final extension cycle of 72°C for 7 min. Products were analyzed by agarose gel electrophoresis. Wild-type animals revealed the smaller 239 bp band, homozygous knock-outs (ERKO) showed the larger 790 bp band, and heterozygotes displayed both bands.

### Neocortical Cultures

Organotypic explant cultures, obtained from 360  $\mu$ m hemispherical slices of the frontal and cingulate neocortex of P2 wild-type and ERKO mice (day of birth = P1), were explanted onto collagen-coated, poly-D-lysine precoated coverslips and maintained in roller tube culture with gonadal steroid-deficient (gelding serum) and phenol red-free nutrient medium, as previously described (Singh et al., 1999, 2000). The nutrient medium was supplemented with 17 $\beta$ -estradiol (2 nM; Sigma, St. Louis, MO) for 1 week, to optimize the development of CNS cultures from estrogen target regions (C. D. Toran-Allerand, unpublished observations).

### Immunoprecipitation and Western Blot Analysis

Tissues were harvested into protease and phosphatase inhibitor-containing lysis buffer (50 mM Tris-base, pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EGTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 5  $\mu$ M ZnCl<sub>2</sub>, 100 mM NaF, 10  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 1 mM PMSF, and 1% Triton X-100) and prepared for immunoprecipitation and polyacrylamide gel electrophoresis, as previously

described (Singh et al., 1999, 2000). Immunoprecipitation was performed, using an indirect technique with magnetic Dynabead separation (Dynal ASA, Oslo, Norway). All procedures were performed at 4°C. In brief, P7 wild-type and ERKO cerebral cortices were homogenized by passing the sample eight times through a syringe fitted with a 20-gauge needle. The homogenate was centrifuged at 100,000  $\times$  g at 4°C for 15 min, and the protein concentration of the supernatant was determined (Lowry's method; Bio-Rad Detergent Compatible Protein Assay kit; Bio-Rad, Hercules, CA). For coimmunoprecipitation experiments, detergent was omitted from the lysis buffer. Depending on the species of the antibodies to be used, the clarified lysates were precleared with either anti-mouse or anti rabbit IgG-coated Dynabeads to reduce nonspecific antibody–antigen binding. For immunoprecipitation of ER-X, the precleared lysates, recovered from the supernatant, were then incubated at 4°C for 12–24 hr with gentle shaking on a Nutator with 6F11, a mouse monoclonal ER antibody raised against the full-length mouse ER- $\alpha$  molecule, which has proven to be optimal for immunoprecipitation of ER-X (Novocastra; Vector Laboratories, Burlingame, CA; 1:50–1:100). Primary antibody incubation was followed by the addition of anti-mouse IgG-coated Dynabeads for 3 hr to capture and precipitate the antibody–antigen complexes. The ER antibodies and coimmunoprecipitated proteins were separated from the Dynabeads by the addition of 1 $\times$  sample loading buffer, containing 5%  $\beta$ -mercaptoethanol, and boiling for 5 min. The Dynabeads were removed from the supernatant, using Dynal Magnetic Particle concentrators. The immunoprecipitated proteins were boiled at 95–100°C for 5 min, and 300–500  $\mu$ g samples were loaded onto 10% SDS-PAGE gels and separated based on molecular size. Prestained rainbow markers (Bio-Rad) were used as molecular mass standards. The gels were then electroblotted onto polyvinylidene difluoride (PVDF) membranes.

Immunodetection of the protein of interest was performed by first blocking the membrane in 5% nonfat dry milk (Carnation; Nestle USA) in TBS-Tween 20 (10 mM Tris-base, 150 mM NaCl, and 0.2% Tween 20, pH 8.0), followed by addition of the primary antibody. Wherever feasible, the PVDF membranes were probed with antibodies different from those used for immunoprecipitation to maximize the specificity of the immunoreactive product obtained. For ER-X in particular, we used either of two antibodies highly specific for ER- $\alpha$ : one specific for the ligand binding domain (LBD) of ER- $\alpha$  (MC20; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA) and the other raised against amino acids 586–600 of the C terminus of ER- $\alpha$  (C1355; 1:2000; Friend et al., 1997; Upstate Biotechnology, Lake Placid, NY). Both antibodies recognize

ER-X on Western immunoblots and by immunohistochemistry, but C1355 is not effective for immunoprecipitation. ER- $\beta$  was identified with antibodies directed against the LBD of ER- $\beta$  (1:250; Zymed, South San Francisco, CA). Negative controls to test for the specificity of the interactions were run in parallel and were performed by immunoprecipitation of the precleared protein lysates with preimmune mouse IgG and subsequently probed with the appropriate antibody. Additionally, a control peptide or lysate (uterus, ovary) was always used as a positive control to verify the identity of the band in the experimental lanes. The specificity of the signal was determined by the apparent molecular weight (MW) of the protein detected.

Antibody binding to protein was detected, using a secondary antibody conjugated to horseradish peroxidase (1:40,000; Pierce, Rockford, IL), and visualized autoradiographically on film, using enzyme-linked chemiluminescence (ECL; Amersham Biosciences, Arlington Heights, IL), as previously described (Singh et al., 1999, 2000). All blots were stripped and reprobed with the appropriate antibody to verify equal loading of protein across lanes and were analyzed densitometrically. For studies of ERK phosphorylation, the blots were first probed with phosphospecific ERK antibodies to detect phospho-ERK1/2 [phospho-p44/42<sup>MAP Kinase</sup>, (Thr202/Tyr204) (1:1000; Cell Signaling, Beverly, MA)]. The same blot was reprobed for total (nonphosphorylated) ERK protein to verify equal loading [ERK-1 (C-16), or ERK-2 (C-14)] (1:1000; Santa Cruz Biotechnology). All antibodies were diluted in the blocking solution.

#### Densitometric Analyses

Densitometric analyses of ERK protein levels were performed to ensure similar levels of protein loaded across lanes. Autoradiograms were scanned in triplicate, using an HP Scanjet 6200C (Hewlett Packard Company, Greeley, CO) and analyzed using Kodak 1D Image Analysis Software (Eastman Kodak, Rochester NY). Net intensity values were calculated by subtracting the background within the area measured for each band from the total intensity within this same measured area to account for any variation in background intensity across the film.

#### CLM Preparation

Membrane fractions were prepared by adapting the detergent-free method of Smart et al. (1995). Briefly, pools of 40–50 P7 ERKO neocortices were homogenized in 20 mM Tricine, pH 7.8, buffer, containing 1 mM EDTA, 0.25 M sucrose, and 1 mM dithiothreitol (TESD buffer), then centrifuged at  $1000 \times g$  at 4°C for 10 min. The pellet was resuspended in TESD buffer, recentrifuged, and the supernatants were pooled. The

combined supernatants were subjected to Percoll gradient fractionation in the same buffer to isolate the plasma membrane fraction. In some binding experiments (indicated below) Percoll-purified plasma membranes were used without further fractionation. For preparation of CLMs, plasma membranes were sonicated and further separated by centrifugation on a linear 20 to 10% OptiPrep (iodixanol) gradient (Nycomed Pharma A. S., Oslo, Norway). Based on their light-buoyant density, CLMs were separated and purified from non-CLMs, using two OptiPrep density gradients. The purity of the CLM preparation was verified immunologically by demonstrating the presence of CLM-enriched proteins: flotillin (1:250), PKC- $\alpha$  and PKC- $\gamma$  (1:1000) (Transduction Labs, Lexington, KY), and absence of the non-CLM-associated cytoskeletal protein paxillin (1:10,000; Transduction Labs). Electrophoretically separated CLMs on PVDF membranes were probed with antibodies specific for ER- $\alpha$  [C1355, (Upstate Biotechnology); MC20 (Santa Cruz Biotechnology)]; ER- $\beta$  (Zymed); flotillin (Transduction Labs) and other caveolar-resident proteins: e.g., PKC- $\alpha$  and PKC- $\gamma$  (Transduction Labs) and noncaveolar-resident proteins: e.g., paxillin (Transduction Labs)].

#### Phosphorylation of ERK1/2 in CLM and Non-CLM Preparations

Phosphorylation of ERK1/2 in ERKO CLM and non-CLM preparations was examined following the method of Liu et al. (1997), except that basal medium Eagle (BME) was used in the place of MEM. Nine parts of CLM or non-CLM preparations were mixed with one part of  $10\times$  BME, pH 7.4, containing BSA 800  $\mu\text{g}/\text{ml}$ , 10 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, leupeptin 100  $\mu\text{g}/\text{ml}$ , soybean trypsin inhibitor 100  $\mu\text{g}/\text{ml}$ , 10 mM MgCl<sub>2</sub>, and 1 mM ATP. For MAP kinase kinase (MEK)1/2 inhibition of ERK activation, the CLMs were pretreated with the MEK1/2 inhibitor, U0126 (10  $\mu\text{M}$ ; Cell Signaling, Beverly, MA) for 30 min before pulsing them with the appropriate estradiol. Aliquots of ERKO CLMs and non-CLMs were exposed for 30 min at 37°C to either 17 $\alpha$ -estradiol (0.1 nM), 17 $\beta$ -estradiol (10 nM), U0126 (10  $\mu\text{M}$ ) or a sham control and processed for ERK1/2 phosphorylation, using antibodies to phosphorylated p44/42<sup>MAP Kinase</sup> (ERK1/2) (Thr202/Tyr204) (Cell Signaling Technology), as previously described (Singh et al., 1999, 2000).

#### Isolation of PNS

To increase the yield of ER-X and to test in a cell-free system whether the presence of ER- $\alpha$  is inhibitory for ERK activation, as we had shown previously in neocortical cultures (Singh et al., 2000), we also studied PNS, a cell-free system that contains all the cell organelles except the nucleus. PNS was isolated from P7

wild-type and ERKO neocortices, according to the method of Smart et al. (1995). Three or four P7 wild-type and ERKO neocortices were homogenized, using a Teflon homogenizer in 1 ml of 20 mM Tricine, pH 7.8, buffer, containing 1 mM EDTA, 0.25 M sucrose, 10  $\mu$ g/ml aprotinin, and 1  $\mu$ g/ml leupeptin. The homogenate was centrifuged at  $1000 \times g$  at 4°C for 10 min. The supernatant obtained is the PNS. The pellet was resuspended in 500  $\mu$ l of the homogenization buffer, recentrifuged, and the PNS obtained was pooled with the first PNS. ERKO and wild-type PNS were mixed with  $10\times$  phosphorylation buffer, and the MAPK assay was performed as described above. PNS samples were exposed to 17 $\alpha$ -estradiol (0.1 nM), 17 $\beta$ -estradiol (10 nM), the ER- $\alpha$ -selective ligand propylpyrazole triol (PPT) (100 nM) (Stauffer et al., 2000) (a gift from J. A. Katzenellenbogen, University of Illinois, Champaign/Urbana), the MEK inhibitor U0126 (10  $\mu$ M), BDNF (100 ng/ml), ethanol (0.001%), DMSO (0.001%), and a sham control; first, for 10 min at 4°C, followed by 10 min at 37°C.

#### Cholesterol Depletion

To determine whether disruption of CLMs impairs estrogen activation of the MAPK cascade, neocortical explants were pretreated on P9 with the sterol binding agent Nystatin (50  $\mu$ g/ml) (Sigma), a compound used extensively to document the association of growth factor receptors with caveolae/CLMs (Huang et al., 1999). This concentration of Nystatin has been shown to result in a significant reduction of cellular cholesterol content without appreciably affecting cell viability (Rothberg et al., 1990). P9 neocortical explants were exposed to Nystatin (50  $\mu$ g/ml) (Sigma), BDNF (100 ng/ml), or vehicle control (PBS) for 1 hr before pulsing with 10 nM 17 $\beta$ -estradiol for 30 min, in the continued presence of inhibitor, BDNF, or vehicle. Explants were then processed by Western immunoblot analysis for phosphoERK expression, using antibodies to phosphorylated p44/42<sup>MAP Kinase</sup> (ERK1/2) (Thr202/Tyr204) (Cell Signaling Technology), as previously described (Singh et al., 1999, 2000).

#### In situ Hybridization

Explants of the ERKO neocortex were processed for in situ hybridization, after 7 d in vitro, by a very sensitive, nonisotopic (digoxigenin) method, using a 48 base oligodeoxyribonucleotide (oligonucleotide) to an  $\alpha$ -specific sequence of the ER- $\alpha$  LBD (BER2), as previously described (Miranda and Toran-Allerand, 1992). Briefly, the probe was 3'-end-labeled with digoxigenin-labeled deoxyuridine triphosphate (dUTP) by terminal deoxynucleotidyl transferase (TdT) (Invitrogen, Grand Island, NY). After hybridization of the synthetic oligonucleotide to the target cDNA, the hybrids were detected by enzyme-linked immunohistochemistry,

using anti-digoxigenin antibodies (Fab fragment), conjugated to alkaline phosphatase (1:500; Boehringer Mannheim, Indianapolis, IN), and an enzyme-catalyzed blue-color reaction (5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium salt).

#### Immunocytochemistry

P7 ERKO and wild-type mice were anesthetized by hypothermia and killed painlessly by transcardial perfusion of saline, followed by 4% paraformaldehyde and 1% glutaraldehyde fixation. The neocortex was processed by pre- and post-embedding immunocytochemistry for ER- $\alpha$  and flotillin, respectively. Sections (50  $\mu$ m) were incubated in anti-ER- $\alpha$  antibodies (C1355, 1:1000; or 6F11, 1:50), washed, and incubated in biotinylated horse-anti-rabbit or anti-mouse IgG, 1:250 (Vector Laboratories), incubated with avidin-biotinperoxidase, 1:50 (Vector Laboratories), and followed by diaminobenzidine (DAB) (brown reaction product). Sections were then processed for electron microscopy, dehydrated, and flat embedded in Durcupan (EM Science, Gibbstown, NJ). Alternate ultrathin sections (Reichert-Jung Ultramicrotome) of the neocortex, immunolabeled for ER- $\alpha$ , were further labeled for flotillin (1:50). Sections were washed and incubated in gold-conjugated (15 nm) goat anti-rabbit IgG (1:20) (EM Science), then washed and contrasted with saturated uranyl acetate. Ultrathin sections were examined using a Philips CM-10 electron microscope.

#### Estrogen Binding Assay

Duplicate aliquots of 1 mg each of protein lysate from ERKO P7 neocortex or wild-type adult uterus were precleared for 30 min, using anti-rabbit IgG-coated magnetic beads (Dynal AS). Precleared protein lysates were immunoprecipitated with anti-ER- $\alpha$  antibodies [6F11 (Novocastra) or MC20 (Santa Cruz Biotechnology)] at 4°C overnight. Immunoprecipitated samples, Percoll-purified plasma membrane fractions, and Optiprep-purified CLM preparations (50  $\mu$ g each) from P7 wild-type or ERKO neocortex were incubated with <sup>3</sup>H-estradiol (2, 4, 6, 7, 16, 17-<sup>3</sup>H-estradiol, 100 Ci/mmol; NEN Life Sciences, Boston, MA) at 4°C for 18 hr. The incubation was terminated by adsorption of the binding sites onto an equal volume of hydroxylapatite (HAP) slurry in TESD buffer. HAP pellets were washed four times with Tris-buffered saline containing 0.2% Tween 20 buffer and extracted with 1 ml of absolute ethanol overnight at room temperature. The ethanol supernatants were transferred to liquid scintillation fluid (5 ml) and counted. Control tubes, used in assessing HAP adsorption of free steroid, contained HAP and the same buffer constituents, without addition of the membranes. Nonspecific binding was assayed in the membranes using the same amount of radioactive ligand plus 200-fold molar excess of unlabeled diethyl-

stilbestrol (DES) (Sigma). Specific binding was calculated by subtracting nonspecific from total binding. The apparent affinity of the membrane binding sites was determined by incubation with a range of concentrations of  $^3\text{H}$ -estradiol (0.25–10 nM). The specificity of the binding sites was studied by coincubation of purified membranes with 2 nM  $^3\text{H}$ -estradiol in the presence of unlabeled progesterone,  $17\alpha$ -estradiol, or  $17\beta$ -estradiol, added at either 25-fold or 500-fold molar excess.

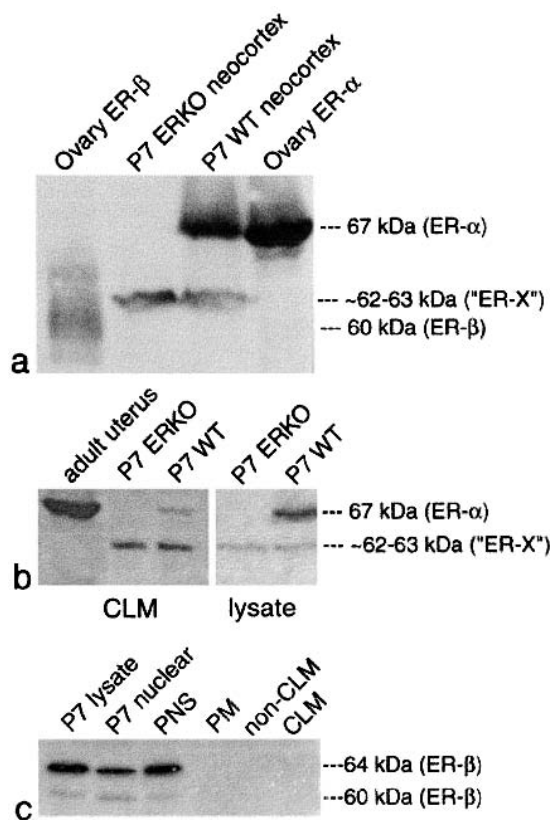
### Transient Cerebral Ischemia Model

Details of the murine model of focal cerebral ischemia, using an intraluminal suture, have been described previously (Huang et al., 2000). Briefly, mice were anesthetized with 0.3 ml of intraperitoneal ketamine (10 mg/ml) and xylazine (0.5 mg/ml) and positioned supine on a rectal temperature-controlled operating surface (Yellow Springs Instruments, Yellow Springs, OH). Animal core temperature was maintained at  $37 \pm 2^\circ\text{C}$  during surgery and for 90 min after surgery. A midline neck incision exposed the right carotid sheath under the operating microscope (Leica). The common carotid artery was isolated and the occipital, pterygopalatine, and external carotid arteries were each isolated, cauterized, and divided. Middle cerebral artery occlusion was accomplished by advancing a 13 mm heat-blunted 6–0 nylon suture via an arteriotomy made in the external carotid stump. After placement of the occluding suture, the external carotid artery was cauterized to prevent bleeding through the arteriotomy, and arterial flow was established. After 45 min the occluding suture was removed, and electrocautery was used to close the arteriotomy. The wound was closed with surgical staples. After 24 hr, the mice were anesthetized, decapitated, and brains were removed intact and placed in a mouse brain matrix (Activational Systems Inc, Warren, MI) for 1 mm sectioning. Sections were immersed in 2% triphenyltetrazolium chloride (Sigma) in 0.9% saline and incubated for 12 min at  $37^\circ\text{C}$ . Infarcted brain was identified as an area of unstained tissue. Slices containing tissue from the region surrounding the infarct (penumbra) and from the comparable region of the noninfarcted hemisphere were processed for immunoprecipitation and Western analysis, using 6F11 and MC20 antibodies to ER- $\alpha$ , respectively. A total of 8 wild-type mice were studied.

### Results

#### P7 Neocortex Contains an ~62–63 kDa Protein That Is Neither ER- $\alpha$ nor ER- $\beta$ and Is Enriched in CLMs of the Plasma Membrane

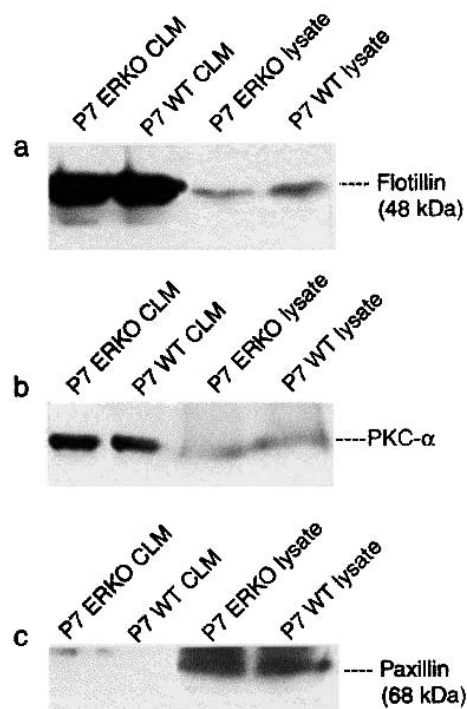
Using antibodies directed against ER- $\alpha$  and ER- $\beta$ , we found, by immunoprecipitation and Western immunoblot analysis of wild-type and ERKO P7 neocortical



**Figure 45.1**

ER-X is neither ER- $\alpha$  nor ER- $\beta$ . *a*, Western immunoblots of P7 wild-type and ERKO neocortex and adult wild-type mouse ovary, using antibodies to the LBDs of ER- $\alpha$  (Santa Cruz Biotechnology; MC-20; ovary and neocortex) and ER- $\beta$  (Zymed; ovary). The apparent MW of ER-X (~62–63 kDa) is clearly different from the MW of the mouse ER- $\alpha$  (67 kDa) and ER- $\beta$  (60 kDa) ovarian controls. *b*, Whereas P7 wild-type neocortex contained both the 67 kDa ER- $\alpha$  and the ~62–63 kDa ER-X bands, P7 ERKO tissues expressed only the ~62–63 kDa ER-X band. P7 wild-type and ERKO neocortical CLM preparations were greatly enriched with the ~62–63 kDa protein. A striking reversal of the ER- $\alpha$ /ER-X ratio was seen in wild-type CLM preparations, in which the ~62–63 kDa form was highly enriched, whereas authentic 67 kDa ER- $\alpha$  was considerably diminished. *c*, Absence of ER- $\beta$  from the plasma membrane, CLM, and non-CLM regions. Note the total absence of ER- $\beta$  from the wild-type plasma membrane and the CLM and non-CLM fractions. Note also the nuclear concentration of the 60 and 64 kDa isoforms of ER- $\beta$ . PM, Plasma membrane; non-CLM, non-caveolar-like membrane; CLM, caveolar-like membrane.

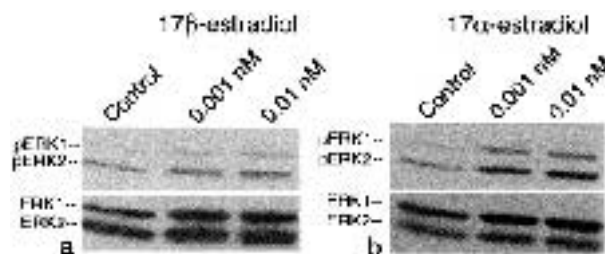
cell lysates, PNS, and CLM preparations, an hitherto unidentified protein, immunoreactive for the LBD of ER- $\alpha$  but not ER- $\beta$ . We propose that this protein represents ER-X. Although immunoreactive for ER- $\alpha$ , this protein has an apparent MW of ~62–63 kDa that is clearly different from that of ovarian ER- $\alpha$  (67 kDa) and ER- $\beta$  (60 kDa) (Fitzpatrick et al., 1999) (figure 45.1a). Cell lysates and detergent-free, highly purified, CLM preparations (Smart et al., 1995) of both P7 neocortical wild-type and ERKO plasma membranes expressed this ~62–63 kDa protein (figure 45.1b). Although P7 wild-type neocortex expressed both the 67 kDa ER- $\alpha$  band and the ~62–63 kDa ER-X band, P7

**Figure 45.2**

Characterization and purity of the CLM preparations. *a*, Western immunoblots of CLMs show enrichment in flotillin, the neuron-specific, integral CLM protein. The purity of CLM preparations was verified by the presence of caveolar-enriched resident proteins such as PKC- $\alpha$  (*b*), and by the absence of the cytosolic protein paxillin, a cytoskeletal component associated with non-CLM regions (*c*).

ERKO neocortex contained only the ~62–63 kDa band. P7 wild-type and ERKO neocortical CLM preparations were greatly enriched with the ~62–63 kDa protein (figure 45.1b). A striking reversal of the 67 kDa/~62–63 kDa ratio was seen in wild-type P7 neocortical CLM preparations, which, although highly enriched in the ~62–63 kDa form, were greatly diminished in the 67 kDa ER- $\alpha$  band. The specificity and significance of the association of the ~62–63 kDa protein with CLMs was emphasized by the failure to detect immunoreactivity for other steroid receptors, such as ER- $\beta$  in CLM, non-CLM, and plasma membrane preparations (figure 45.1c), although its presence was clearly demonstrable in P7 neocortical cell lysates and in the nuclear fraction and PNS (figure 45.1c).

The purity of the CLM preparations was verified by demonstrating the presence of the CLM integral protein flotillin (Bickel et al., 1997) (figure 45.2a) and such CLM-enriched resident proteins as PKC- $\alpha$  (figure 45.2b) and PKC- $\gamma$  (data not shown) (Smart et al., 1995), and by the absence of the cytosolic protein paxillin (figure 45.2c), a cytoskeletal component associated with non-CLM regions of plasma membranes (Smart et al., 1995).

**Figure 45.3**

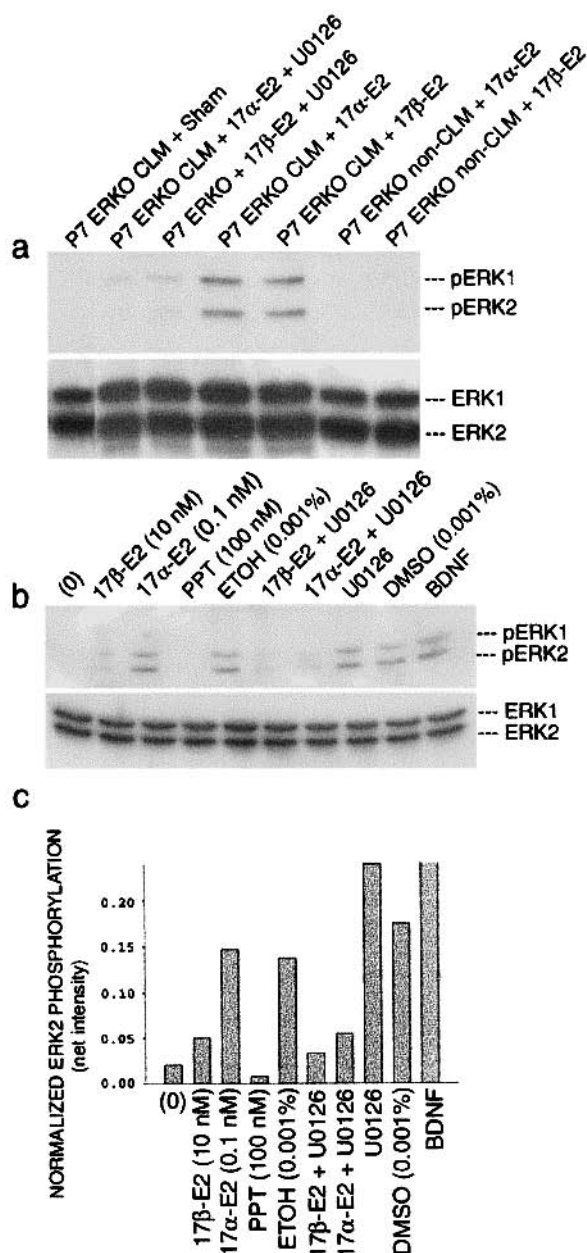
ER-X is exquisitely sensitive to picomolar concentrations of 17 $\alpha$ - and 17 $\beta$ -estradiol. Western immunoblot of ERK1/2 phosphorylation elicited in wild-type neocortical explants by 17 $\beta$ -estradiol (*a*) and 17 $\alpha$ -estradiol (*b*). *Bottom blots*, Reprobing with antibodies to total nonphosphorylated ERK1/2 to verify equal loading of ERK1/2 protein across lanes. pERK, phosphoERK. Densitometry confirmed equal loading. Note that significantly higher levels of 17 $\beta$ -estradiol were required for ERK activation, perhaps reflecting the need in wild-type cultures to overcome the inhibitory effect of ER- $\alpha$  on ERK phosphorylation, which, unlike 17 $\alpha$ -estradiol, 17 $\beta$ -estradiol activates as well.

#### ER-X Has an Entirely Different Steroid Specificity than Either ER- $\alpha$ or ER- $\beta$

The steroid specificity for estrogen-induced activation of ERK1/2 phosphorylation is radically different from that of either ER- $\alpha$  or ER- $\beta$ : ERK1/2 is not activated by either ER- $\alpha$ -selective ligands such as 16 $\alpha$ -iodo-17 $\beta$ -estradiol (Singh et al., 2000) and PPT (Stauffer et al., 2000) (100 nM) (see figure 45.4b,c) or by ER- $\beta$ -selective ligands such as genistein and coumestrol (Singh et al., 2000) but is activated equally well by picomolar concentrations of 17 $\alpha$ - and 17 $\beta$ -estradiol (figure 45.3a,b). In wild-type cultures 17 $\alpha$ -estradiol, a natural stereoisomer of 17 $\beta$ -estradiol that is generally considered to be transcriptionally inactive, elicited a stronger, sustained activation of ERK1/2 at the 1–10 pM ( $10^{-12}$  M) range (figure 45.3b) than did 17 $\beta$ -estradiol (0.1–10 nM) (figure 45.3a). What makes this response so astonishing is that 17 $\alpha$ -estradiol, which, like 17 $\beta$ -estradiol, is derived from aromatization of androgens, but whose site of synthesis is unclear, has a 100-fold lower affinity for ER- $\alpha$  than 17 $\beta$ -estradiol (Hajek et al., 1997). Significantly, higher levels of 17 $\beta$ -estradiol were required for ERK activation in wild-type neocortical cultures (figure 45.3a), perhaps reflecting the need to overcome the inhibitory effect of ER- $\alpha$  on ERK1/2 phosphorylation (Singh et al., 2000) (figure 45.4), which, unlike 17 $\alpha$ -estradiol, 17 $\beta$ -estradiol activates as well. That the inhibitory presence of ER- $\alpha$  influences dose responsiveness is suggested by observation that in the ER- $\alpha$ -deficient ERKO neocortical explants, 17 $\beta$ -estradiol, like 17 $\alpha$ -estradiol, is also able to elicit activation of ERK in the 1–10 pM range (data not shown).

#### Estrogen Elicits ERK1/2 Activation in CLMs

To provide direct evidence that the CLM-associated ~62–63 kDa ER-X protein is connected with

**Figure 45.4**

Estrogen-induced activation of ERK1/2 in CLMs and PNS. Western immunoblots: *a*, exposure of highly purified, P7 ERKO neocortical CLMs to 17 $\alpha$ -estradiol (0.1 nM) and 17 $\beta$ -estradiol (10 nM) for 30 min elicited MEK-dependent (U0126) phosphorylation of ERK1 and ERK2. Non-CLM regions were unresponsive. Densitometry confirmed equal loading of protein. *b*, Exposure of P7 wild-type neocortical PNS to 17 $\alpha$ -estradiol (0.1 nM) and 17 $\beta$ -estradiol (10 nM) for 10 min, 37°C elicited MEK-dependent (U0126) phosphorylation of ERK1 and ERK2. Note that, not only did the ER- $\alpha$ -selective ligand PPT reduce ERK phosphorylation levels below baseline (0) very significantly, but that the level of ERK1/2 phosphorylation, elicited by 17 $\beta$ -estradiol, was also significantly lower than after exposure to 17 $\alpha$ -estradiol. This difference may be attributed to the fact that P7 wild-type neocortex is also enriched in ER- $\alpha$  which, because it is activated by 17 $\beta$ - (but not 17 $\alpha$ -) estradiol and exerts its inhibitory effect on ERK1/2, as was also seen after exposure to PPT. *Bottom blots*, Reprobing with antibodies to nonphosphorylated ERK1/2 to verify equal loading of ERK protein across lanes. Densitometry confirmed equal loading. *c*, Densitometric analysis of ERK activation in wild-

estrogen-induced ERK1/2 activation, we showed that exposure of highly purified, P7 ERKO neocortical CLMs to 17 $\beta$ -estradiol (10 nM) and 17 $\alpha$ -estradiol (0.1 nM) for 30 min elicited phosphorylation of ERK1/2 (figure 45.4a). In both instances ERK activation was inhibited by the MEK inhibitor U0126 (figure 45.4a). In contrast, non-CLM regions of the plasma membrane, exposed similarly, did not respond (figure 45.4a).

#### ER- $\alpha$ Is an Inhibitory Regulator of ERK1/2 Activation in PNS

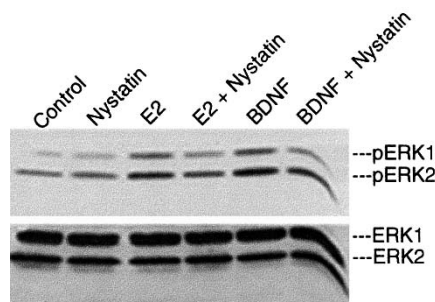
We then investigated in wild-type PNS, a cell-free system, whether ER- $\alpha$  is an inhibitory regulator of estrogen-induced ERK1/2 activation, as we had shown previously in neocortical explants (Singh et al., 2000). Using the ER- $\alpha$ -selective ligand PPT (Stauffer et al., 2000) (100 nM) in wild-type neocortical PNS, we found that MEK-inducible ERK1/2 phosphorylation was dramatically reduced below baseline (figure 45.4b). Of particular note, furthermore, were the findings that the levels of 17 $\beta$ -estradiol-induced ERK1/2 phosphorylation were significantly less than after exposure to 17 $\alpha$ -estradiol, although both were inhibited by the MEK inhibitor U0126. This difference in responsiveness may be attributed to the fact that, at P7, wild-type neocortex is also enriched with maximal levels of ER- $\alpha$  (Gerlach et al., 1983) which, when activated by 17 $\beta$ - (but not 17 $\alpha$ -) estradiol, is able to exert its inhibitory effect on ERK1/2, as is also seen after exposure to PPT. These findings confirm that ER- $\alpha$  is a strong inhibitor of ERK1/2 activation, a measure of which is given by the ability of PPT to effectively prevent activation of ERK1/2 even in the face of strong ERK1/2 activation, elicited by the PPT vehicle ethanol (figure 45.4b,c). These findings provide not only additional proof that ER- $\alpha$  does not mediate activation of the MAPK cascade but also compelling evidence confirming the role of ER- $\alpha$  as an inhibitory modulator of ERK1/2 activation.

#### Cholesterol Disruption in CLMs Decreases Estrogen

##### Activation of ERK

CLMs, like caveolae, are highly enriched in cholesterol, glycosphingolipids, sphingomyelin, and lipid-anchored membrane proteins, which serve as multivalent scaffolding onto which many signaling kinases assemble to generate preassembled signaling complexes. Eighty to ninety percent of plasma membrane cholesterol is concentrated within caveolae/CLMs, where it plays a critical role in maintaining receptor

type PNS shown in *b*. These findings confirm that ER- $\alpha$  is a strong inhibitor of ERK activation, a measure of which is shown by the ability of PPT to effectively prevent ERK activation even in the face of the strong activation of ERK elicited by the PPT vehicle ethanol.



**Figure 45.5**

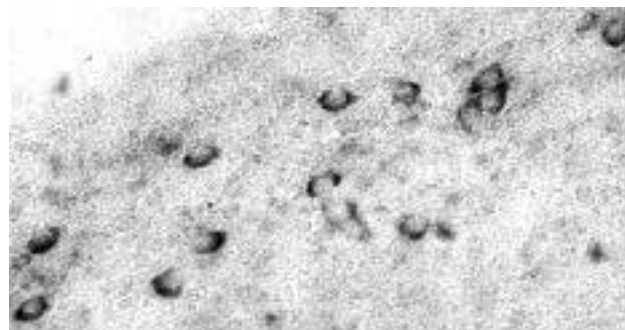
Disruption of cholesterol in CLMs impairs ERK activation. Selective disruption of membrane cholesterol by Nystatin in 9-d-old wild-type neocortical explants decreased the ability of estradiol and the BDNF control to elicit ERK phosphorylation. *Bottom blots*, Reprobing with antibodies to nonphosphorylated ERK1/2 to verify equal loading of ERK protein across lanes. Densitometry confirmed equal loading.

protein association within the CLM domain (Rothberg et al., 1990). The sterol-binding agent Nystatin has been used extensively to document the association of growth factor receptors with caveolae/CLMs (Huang et al., 1999). To determine whether selective disruption of cholesterol in CLMs impairs the ability of estrogen to elicit ERK1/2 phosphorylation, we exposed P9 neocortical explants to Nystatin (50  $\mu$ g/ml) for 1 hr before pulsing with 17 $\beta$ -estradiol (10 nM), BDNF (100 ng/ml), or the vehicle control (PBS) for 30 min (figure 45.5) and then processing for ERK1/2 phosphorylation by Western blot analysis. We found that disruption of membrane cholesterol decreased the ability of both estradiol and BDNF to elicit ERK1/2 phosphorylation, providing additional evidence of the contributions of CLMs to estradiol-induced ERK1/2 activation.

#### ER-X Has Homology with ER- $\alpha$ LBD and Is Expressed in the Plasma Membrane

Using an oligonucleotide probe directed against an  $\alpha$ -specific region of the ER- $\alpha$  LBD (BER2) (Miranda and Toran-Allerand, 1992), we found widespread distribution of the blue ER- $\alpha$ -like hybridization signal in neurons of cultured slices of the ER- $\alpha$ -deficient P2 ERKO neocortex, 17 d in vitro (figure 45.6). This pattern of hybridization in ERKO neocortex suggests that, in view of the absence of ER- $\alpha$ , the oligonucleotide sequence used may share some homology with ER-X mRNA.

Direct evidence that ER-X may be a neuronal plasma membrane-associated ER protein with some homology to ER- $\alpha$  was also obtained in the ERKO neocortex by means of light and electron microscopic immunohistochemistry (figure 45.7a–e). Using polyclonal antibodies, generated against the final 14 C-terminal amino acids of the rat ER and highly specific for ER- $\alpha$  (C1355, Upstate Biotechnology) (Schreihöfer et al., 1999), large numbers of immature ERKO neo-



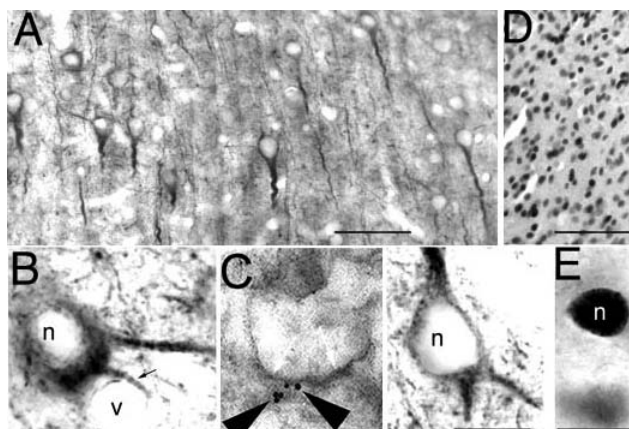
**Figure 45.6**

ER-X has homology with the LBD of ER- $\alpha$ . Whole-mount of a P2 ERKO neocortical explant, 17 d in vitro. The culture was stained for ER- $\alpha$  mRNA by in situ hybridization with a 48 base oligonucleotide probe to an  $\alpha$ -specific region of the ER- $\alpha$  LBD (BER2; Miranda and Toran-Allerand, 1992) and shows the ER- $\alpha$ -like mRNA hybridization signal in neocortical neurons. Residual, untranslated ER- $\alpha$  mRNA? A splice variant of ER- $\alpha$  mRNA? Or the mRNA for a novel ER, ER-X?

cortical neurons with unstained nuclei were seen (figure 45.7a,b). Immunoreactivity was clearly localized to the cell membrane and cytoplasm and not in the nucleus. In figure 45.7b, a blood vessel (V) is in close proximity to a labeled dendrite, an association which suggests a mechanism by which estrogen could get even more efficiently onto ER-X. On the other hand, using monoclonal antibodies generated against full-length mouse ER- $\alpha$  (6F11; Novocastra) (figure 45.7D,E) and said to recognize the 5' N terminus region, the opposite result was obtained: nuclear labeling was observed but no cytoplasmic or membrane labeling was seen. Because we have found that 6F11 cross-reacts significantly with ER- $\beta$  by Western blotting (data not shown), the nuclear labeling observed here most likely reflects intranuclear ER- $\beta$ , which is normally expressed in both wild-type and ERKO neocortex. Association of the ~62–63-kDa protein with CLMs was further documented at the ultrastructural level on ultrathin cryostat sections of P7 ERKO neocortex by demonstrating immunoreactive flotillin, labeled by gold particles, colocalized with horseradish peroxidase-labeled immunoreactivity for ER- $\alpha$  on a neocortical dendritic spine (figure 45.7C).

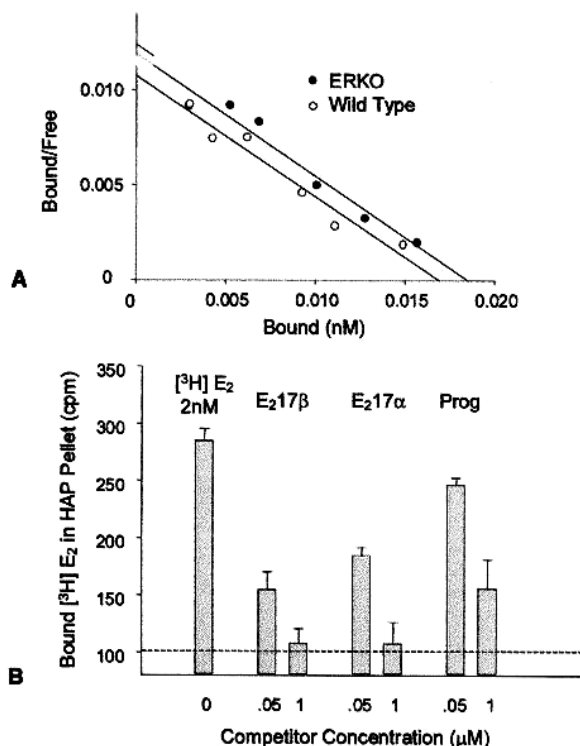
#### ERKO Neocortical Plasma Membranes Contain an Estrogen-Binding Protein (ER-X)

We determined that neocortical plasma membranes contain a unique estrogen-binding protein by scintillation counting of  $^3$ H-estradiol binding to the ~62–63-kDa ER-X protein in highly purified P7 ERKO CLM preparations. In these preparations, the only detectable ER-immunoreactive material present was the ~62–63-kDa protein (figure 45.1). Binding of 10 nM  $^3$ H-estradiol to P7 ERKO CLMs appeared to be specific and saturable, in that it was suppressed in the presence

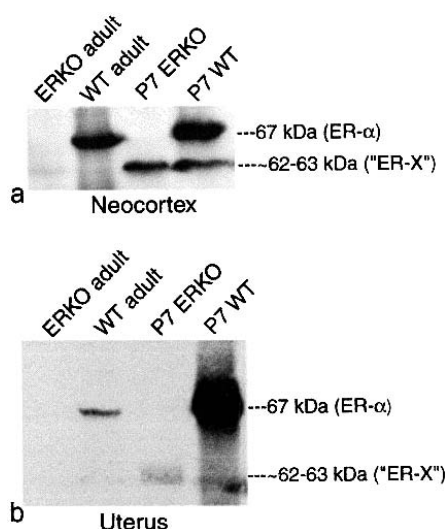
**Figure 45.7**

Direct evidence in ERKO that ER-X is a neuronal plasma membrane-associated receptor with some homology to the ER- $\alpha$  LBD. *A*, Using antibodies highly specific for an  $\alpha$ -specific region of the LBD of ER- $\alpha$  (C1355), large numbers of immature immunoreactive neocortical ERKO neurons with unstained nuclei are seen. *B*, The immunoreactivity is clearly localized to the cell membrane and cytoplasm and not in the nucleus. *D, E*, Antibodies, raised against the full-length ER- $\alpha$  molecule, said to recognize epitopes in the 5', N-terminal region (6F11), but which we have found also to cross-react significantly with ER- $\beta$ , show widespread nuclear labeling with no cytoplasmic or membrane labeling seen. The nuclear labeling observed most likely reflects intranuclear ER- $\beta$ , which is normally expressed in both wild-type and ERKO neocortical neurons. *C*, CLM association of ER-X in ERKO neocortical neurons was further documented at the ultrastructural level by demonstrating immunoreactive flotillin (gold particles), colocalized with immunoreactivity for the ER- $\alpha$  LBD (horseradish peroxidase) on a mushroom-like neocortical dendritic spine. Scale bars, 10  $\mu$ m.

of unlabeled diethylstilbestrol (DES). Neocortical CLM preparations from P7 ERKO mice, shown to be highly enriched in ER-X, were similarly highly enriched in DES-sensitive estrogen binding (282.12 fmol/mg CLM protein), as compared with P7 ERKO neocortical lysates (9.94 fmol/mg lysate protein) and wild-type adult uterine lysates (38.85 fmol/mg lysate protein). Further characterization of the membrane binding sites was achieved using Percoll-fractionated plasma membranes, containing both CLM and non-CLM components, to increase the yield of total membrane sufficiently to allow construction of binding isotherms and performance of specificity studies. In Percoll-purified membranes from P7 ERKO neocortices, as in CLMs, the only detectable ER-immunoreactive protein present was the ~62–63-kDa band (data not shown). Membranes from both P7 ERKO and P7 wild-type neocortex contained a high-affinity, saturable  $^3$ H-estradiol binding site ( $K_d$ , ~1.6 nM) (figure 45.8A). Addition of 50 nM unlabelled  $17\beta$ -estradiol or  $17\alpha$ -estradiol markedly inhibited binding of  $^3$ H-estradiol. In the presence of a 1  $\mu$ M concentration of either estrogen, binding of the tritiated ligand was reduced to the nonspecific levels observed in the presence of excess DES (figure 45.8B). Unlabelled progesterone, by con-

**Figure 45.8**

Binding of  $^3$ H-estradiol to Percoll-purified plasma membranes from P7 ERKO and wild-type mouse neocortex. *A*, Identical amounts of membrane protein (50  $\mu$ g/tube) were incubated with varying concentrations of  $^3$ H-estradiol (0.3–8 nM) for 18 hr at 4°C. The reaction was terminated by addition of hydroxylapatite (HAP). The membranes and HAP were sedimented by centrifugation in a microfuge, and the pellet was washed four times to remove free steroid. Radioactivity in the pellets was extracted with ethanol and counted. Nonsaturable binding, assessed in the presence of 1  $\mu$ M unlabeled DES, was subtracted from the total counts, and the saturable binding was plotted as the ratio of bound–unbound ligand versus the concentration of bound  $^3$ H-estradiol. Similar concentrations of high-affinity binding (equilibrium dissociation constant,  $K_d$ , ~1.6 nM) were observed in wild-type and ERKO membranes. *B*, Specificity of the binding site in Percoll-purified membranes from P7 ERKO mouse neocortex. Aliquots of plasma membrane were incubated with 2 nM  $^3$ H-estradiol for 18 hr at 4°C in the presence and absence of different concentrations (50 nM and 1  $\mu$ M) of  $17\alpha$ -estradiol,  $17\beta$ -estradiol, or progesterone. Bound  $^3$ H-estradiol was separated by sedimentation with HAP and counted at an efficiency of 50%. Data represent the number of bound counts (after subtraction of HAP-only blank control tubes, containing no membrane protein) expressed as the means  $\pm$  SD of triplicate determinations. The horizontal dashed line indicates the level of nonspecific binding observed in the presence of 1  $\mu$ M DES.



**Figure 45.9**

ER-X is developmentally regulated. ER-X expression is developmentally regulated and is maximally expressed at ~P7–P10 in the neocortex (a) and the uterus (b). During the first postnatal month, wild-type and ERKO neocortical ER-X levels decline dramatically and become barely visible in the adult.

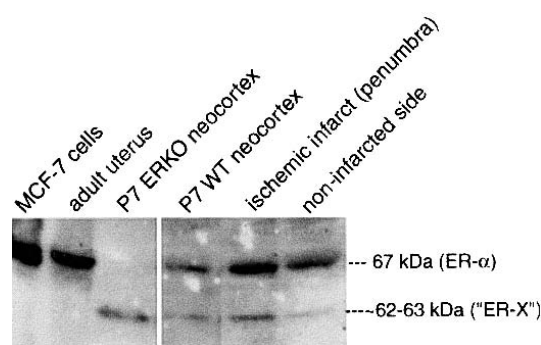
trast, was less effective than either estrogen, progesterone only partially suppressing binding of  $^3\text{H}$ -estradiol when added in 500-fold molar excess (figure 45.8B).

#### ER-X Is Developmentally Regulated in the Brain and Uterus

Expression of the ~62–63 kDa ER-X protein is developmentally regulated and is maximally expressed ~P7–P10 in both the neocortex and uterus (figure 45.9a,b). During the first postnatal month, wild-type and ERKO neocortical and uterine levels of the ~62–63 kDa protein declined until P21 and became dramatically reduced in the adult, which expressed little of this protein.

#### ER-X Is Upregulated in a Rodent Model of Brain Injury

To test whether re-expression of the developmentally regulated ER-X might return after brain injury in the adult, as has been reported for the developmentally regulated ER- $\alpha$  (Dubal et al., 2001), we analyzed a mouse ischemic stroke model, elicited by transient intraluminal middle cerebral artery occlusion (Huang et al., 2000). Tissue from the region surrounding the infarct (the penumbra) was compared with the comparable region of the noninfarcted neocortex of the opposite side, 24 hr after occlusion. Using immunoprecipitation, followed by Western blotting, we found upregulation of the ~62–63-kDa protein in the penumbra (figure 45.10) to levels comparable with those present during development, as compared with the noninfarcted side that remained unchanged. There was also upregulation of ER- $\alpha$  (figure 45.10), as has been shown previously (Dubal et al., 2001).



**Figure 45.10**

ER-X is upregulated after ischemic brain injury in the adult. Comparison of ER- $\alpha$  and ER-X expression in the infarcted and non-infarcted adult neocortex. After a large ischemic infarct in the neocortex produced by middle cerebral artery occlusion, there was not only upregulation of ER- $\alpha$  expression in the penumbra of the ligated, ischemic side but also upregulation of ER-X therein as well, suggesting re-expression of a developmental mechanism normally latent in the adult. Note the lack of significant ER-X expression on the noninfarcted side. MCF-7 mammary tumor cells and adult uterus = ER- $\alpha$  controls; P7 neocortex = ER-X control.

#### Discussion

These data point strongly to the existence of a novel, plasma membrane-associated, putative estrogen receptor (ER-X). Although membrane ERs have been identified immunologically as ER- $\alpha$  in several cell and tissue systems (Blaustein, 1992; Watson et al., 1999; Razandi et al., 1999; Milner et al., 2001), our findings suggest that ER-X is a unique, functionally distinct, and hitherto unidentified receptor, based on its MW, ligand specificity, cellular localization, and apparent response characteristics. Although ER-X reacts with antibodies to the ER- $\alpha$  LBD, ER-X is not membrane-associated ER- $\alpha$ . Its apparent MW of ~62–63 kDa is clearly different from that of both ER- $\alpha$  (67 kDa) and ER- $\beta$  (60 kDa) (figure 45.1a). Although a functional isoform of ER- $\beta$  with an additional 18 amino acids inserted in the LBD has been identified in rat and mouse tissues (ER- $\beta$ 2) (Petersen et al., 1998), ER-X cannot represent ER- $\beta$ 2, because (1) antibodies directed against the ER- $\alpha$  LBD cross-react with ER-X and do not recognize intranuclear ER- $\beta$ . (2) The anti-ER- $\beta$  antibody we used (Zymed) does not react with ER- $\alpha$  but does cross-react on Western blots with the molecular isoforms of rat ER- $\beta$  observed in tissue lysates (N. J. MacLusky and I. S. Nethrapalli, unpublished observations): no immunoreactivity was detected with this antibody in blots from CLMs enriched in ER-X. (3) ERK1/2 is not activated by ER- $\alpha$  or ER- $\beta$ -selective agonists (Singh et al., 2000) (figure 45.4b). (4) Unlike ER- $\alpha$  or ER- $\beta$ , ER-X is not stereospecific, responding equally well to picomolar concentrations of 17 $\alpha$ - and 17 $\beta$ -estradiol (figure 45.3a,b), whereas

ER- $\alpha$  and ER- $\beta$  exhibit a markedly higher affinity for 17 $\beta$ - than for 17 $\alpha$ -estradiol (Kuiper et al., 1997).

ER-X is part of a multimolecular CLM complex, comprising immunoreactivity for ER- $\alpha$  (but not ER- $\beta$ ) in association with hsp90, members of the MAPK cascade (Toran-Allerand et al., 1999; Singh et al., 1999; Toran-Allerand, 2000) and flotillin, the multivalent, 48-kDa scaffolding protein and neuronal homolog of the caveolar protein caveolin (Bickel et al., 1997). Two recent studies (Levin, 2002; Razandi et al., 2002), published after this paper was submitted, reported association of ER- $\alpha$  immunoreactivity with caveolae in vascular and breast cancer (MCF-7) cells. Although caveolin-associated ER was identified by the authors as ER- $\alpha$  (Razandi et al., 2002), the MW of the immunoreactive band was stated to be 62 kDa, not 67 kDa, as would be expected for authentic full-length ER- $\alpha$ . Rather than demonstrating caveolar association of ER- $\alpha$ , as Razandi et al. (2002) concluded, these data are consistent with the observations presented here. In vascular and MCF-7 cells, like neuronal CLMs, caveolar-associated ER- $\alpha$  immunoreactivity represents primarily a protein with an apparent MW  $\sim$ 5 kDa less than that of authentic ER- $\alpha$ . In brain, both P7 wild-type and ERKO neocortical CLM preparations were greatly enriched with the immunoreactive  $\sim$ 62–63-kDa ER-X protein (figure 45.1b) and depleted of ER- $\alpha$  and ER- $\beta$  (figure 45.1b,c), supporting the selectivity and specificity of the ER-X association with CLMs.

Surprisingly, in both wild-type and ERKO neocortical explants and CLMs, 17 $\alpha$ -estradiol, the natural stereoisomer of 17 $\beta$ -estradiol with 100-fold lower affinity for ER- $\alpha$ , (Hajek et al., 1997) also elicited sustained MEK-dependent activation of ERK1/2 in the picomolar range (figures 45.3b, 45.4a,b). We showed earlier that ER- $\alpha$ - and ER- $\beta$ -selective ligands failed to elicit ERK1/2 activation in wild-type neocortical explants (Singh et al., 2000) and suggested that ER- $\alpha$  may be an inhibitory regulator of ERK activation. This has been confirmed in the PNS cell-free system (figure 45.4b,c). The absence of an inhibitory response in ERKO PNS (data not shown) is consistent with the absence of authentic 67 kDa ER- $\alpha$  from ERKO brains.

Nystatin disrupts cholesterol in cell membranes (Iwabuchi et al., 2000) by forming globular deposits that alter the planar organization of the membrane (McGookey et al., 1983), thereby selectively inhibiting caveolar trafficking without altering other cell functions such as clathrin-mediated endocytosis (Ros-Baro et al., 2001) or intracellular receptor trafficking back to the cell surface (Subtil et al., 1999). Nystatin (50  $\mu$ g/ml) has been shown to significantly reduce cellular cholesterol content without appreciably affecting cell viability. This concentration of Nystatin impaired estradiol induced ERK1/2 activation (figure 45.5).

The existence of plasma membrane-associated ERs (Pietras and Szego, 1977) has been controversial because of previous failures to isolate and characterize such a membrane-associated receptor. Hypothetical mechanisms have included plasma membrane versions of classical intranuclear ER- $\alpha$  and ER- $\beta$  (Blaustein, 1992; Watson et al., 1999; Razandi et al., 1999; Milner et al., 2001), novel members of the ER family (Das et al., 1997; Gu et al., 1999; Nadal et al., 2000), G-protein-coupled receptors (Kelly and Wagner, 1999; Filardo et al., 2000; Wyckoff et al., 2001), or even growth factor-like receptor tyrosine kinases (Anuradha et al., 1994).

That ER-X may have sequence homology with the ER- $\alpha$  LBD was suggested by (1) the strong hybridization signal obtained in ERKO neocortical explants with an oligonucleotide probe specific for the ER- $\alpha$  LBD (Miranda and Toran-Allerand, 1992) (figure 45.6) and (2) ER- $\alpha$ -like immunoreactivity in ERKO neocortex, using antibodies to the ER- $\alpha$  LBD (figure 45.7A,B) but not with those recognizing the N-terminal region (figure 45.7D,E). To generate ERKO, the ER- $\alpha$  gene was disrupted by insertion of a 1.8-kb PGK-Neomycin sequence in the region of exon 2,  $\sim$ 280 bp downstream of the transcription start codon (N terminus) (Lubahn et al., 1993), a region far upstream from the LBD (exons 4–8). Therefore, ER- $\alpha$ -like mRNA found in ERKO neocortex may represent either (1) residual, untranslated ER- $\alpha$  mRNA, (2) a splice variant of ER- $\alpha$ , or (3) ER-X mRNA itself. Residual, weak estrogen binding not attributable to ER- $\beta$  has been reported in both ER- $\alpha$  (ERKO) and ER- $\alpha$ /ER- $\beta$ - (double) knockout adult mouse brains (Shughrue et al., 2002). This binding was identified in ERKO only as a splice variant of ER- $\alpha$  at exon 2 that may regulate the progesterone receptor. Nonetheless, there are compelling reasons to believe that ER-X does not represent the protein product of such a splice variant. A splice variant at exon 2 would contain exactly the same LBD sequence as authentic ER- $\alpha$ . However, the ligand specificity of ER-X is clearly different from that of ER- $\alpha$  in that ER-X responds equally well to picomolar concentrations of 17 $\alpha$ - and 17 $\beta$ -estradiol (figure 45.3a,b). Finally, ER-X simply cannot represent expression of a protein derived from the targeted gene disruption, used to generate ERKO mice, because ER-X is present at comparable levels in P7 wild-type and ERKO neocortex (figure 45.1b). Earlier studies of cellular variations in ER mRNA translation (Toran-Allerand et al., 1992) have provided data consistent with the hypothesis that some of the ER- $\alpha$ -like mRNA detected by *in situ* hybridization may actually represent ER-X mRNA. Although estrogen binding and ER mRNA expression always colocalized, neurons expressing ER mRNA did not always exhibit nuclear binding, and there was no

clear-cut relationship between the widespread hybridization signal (Miranda and Toran-Allerand, 1992) and the limited extent of estrogen binding (Gerlach et al., 1983); evidence of ER-X mRNA?

Our data do not prove that the ~62–63-kDa ER- $\alpha$ -immunoreactive protein binds estrogen. The SDS-PAGE conditions required to separate the ~62–63-kDa protein are incompatible with retention of binding site integrity. Nevertheless, circumstantial evidence suggests that the ~62–63-kDa protein binds estradiol and, moreover, that this binding reaction may mediate activation of ERK1/2. The ~62–63-kDa band and the estradiol binding site are both present in P7 ERKO neocortical membranes that contain neither ER- $\alpha$  nor ER- $\beta$ . In ERKO mouse neocortex, 17 $\alpha$ - and 17 $\beta$ -estradiol both activate ERK1/2: both also compete strongly for membrane binding of <sup>3</sup>H-estradiol (figure 45.8). Levels of membrane binding are similar in ERKO and wild type neocortex, consistent with the observation that similar concentrations of the ~62–63-kDa-immunoreactive band are present in membranes from ERKO and wild-type P7 mice (figure 45.1). Finally, progesterone, which does not bind intranuclear ER- $\alpha$  or ER- $\beta$  but does activate ERK in developing brain (Singh, 2001), is capable of competing with <sup>3</sup>H-estradiol for the membrane binding site, albeit less effectively than 17 $\alpha$ - and 17 $\beta$ -estradiol.

ER-X expression is developmentally regulated in both neocortex and uterus and is maximally expressed at ~P7–P10. Wild-type and ERKO neocortical and uterine ER-X levels declined during the first postnatal month and became dramatically reduced in the adult, which expressed little ER-X (figure 45.9a,b). Transient, neocortical expression of ER-X mimics the developmental pattern of estrogen binding (Gerlach et al., 1983). That an ER other than ER- $\alpha$  may have developmental importance is even suggested by the original ERKO paper (Lubahn et al., 1993). Because loss of functional ER- $\alpha$  did not appear to influence prenatal sexual development, the authors concluded that development of the reproductive tract can occur in the absence of ER-mediated responsiveness. An alternate explanation is that early development may depend on another ER, such as ER-X.

Developmentally regulated estrogen receptors may be upregulated and re-expressed in the adult brain. Previous studies have demonstrated that 17 $\alpha$ - and 17 $\beta$ -estradiol protect against ischemic CNS injury, as well as neuronal cell death induced by exposure to peroxides or  $\beta$ -amyloid (for review, see Green and Simpkins, 2000). The neuroprotective efficacy of 17 $\alpha$ -estradiol has been interpreted as evidence of a direct antioxidant, as opposed to an ER-dependent, mechanism (Behl et al., 1997; Green et al., 1997). Our results suggest an alternative explanation: responses to 17 $\alpha$ -estradiol may, in part, reflect activation of membrane ER-X response

pathways. Developmentally regulated ERs, such as neocortical ER- $\alpha$  and ER-X, latent in the brain since development, may be re-expressed in the adult after injury caused by ischemia, loss of trophic support, or steroid deprivation. ER-X and its signaling pathways could therefore underlie not only the differentiative effects of estrogen in the developing brain but some of its neuroprotective actions in the adult (Simpkins et al., 1997; Dubal et al., 1998; Green and Simpkins, 2000).

Proof that ER-X is a novel ER must await sequencing of the protein, currently in progress. The data presented here, however, suggest that this protein is functional and is associated with estradiol-induced activation of the MAPK cascade. Our data further suggest that responses to estrogen during development and after injury are not necessarily mediated via either ER- $\alpha$  or ER- $\beta$ . Association with CLMs positions ER-X uniquely to interact with colocalized signaling kinases, providing a novel mechanism for mediation of the influences of estrogen on neuronal differentiation (Toran-Allerand, 1976), survival (Garcia-Segura et al., 2001), and plasticity (Matsumoto and Arai, 1981).

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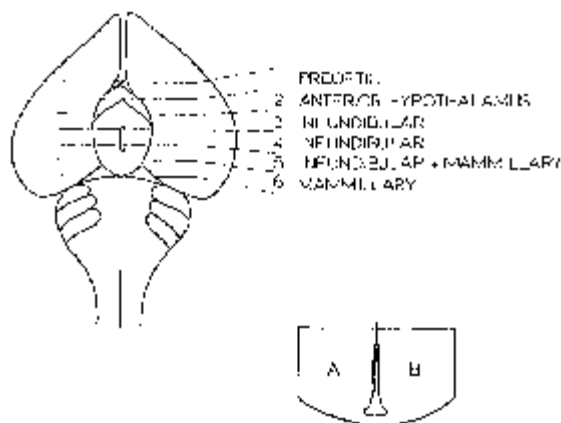
Sexual differentiation of the neural control of reproductive function in mammals is thought to result from exposure of the brain to testicular androgens during a critical period of neural differentiation (for review, see ref. 5). In the rodent, this occurs in the early neonatal period, during the first 5 postnatal days. The functional consequences of neonatal androgenization (or of excessive estrogen) of the rodent brain have led to the concept that, regardless of genetic sex, the newborn brain, though sexually undifferentiated and bipotential, is intrinsically organized to support the cyclical or female pattern of reproductive activity. How androgen elicits this irreversible effect and which neural circuits are involved are poorly understood. Increasing evidence, however, suggests that the initial mode of androgen action may involve its intraneuronal aromatization to estrogen (14) in such neural sites as the preoptic area, hypothalamus and the amygdala. These regions are poorly differentiated at birth and the onset of both structural and functional maturation in at least one of them, the preoptic area, has been shown to coincide with the end phase of the critical period (20). Raisman and Field (17), moreover, have shown in the adult rodent the existence of sexual dimorphism in the synaptic organization of the preoptic area and its dependence on neonatal androgen. Despite this demonstration of plasticity and reorganization of neuropil during the critical period, the morphogenetic aspects of and anatomic basis for sexual differentiation of these limbic regions remain essentially unknown.

The aim of the present study was to investigate the morphogenetic effects of the gonadal steroids (estrogen and androgen) on the development of organotypic cultures of the newborn mouse hypothalamus/preoptic area. This report presents, for the first time, morphological evidence of a direct effect of the sex steroids on the in vitro developmental patterns of neurites from selected areas of these regions. These observations suggest that differential axonal growth rates may be an important factor in the neurogenesis of sexual differentiation of the brain.

The hypothalamus of sexed, newborn mice was cut into 6 coronal sections approximately 300  $\mu\text{m}$  thick

(figure 46.1). Because of regional anatomic differences and because of developmental disparity between littermates and within the hypothalamus proper, each section was then halved mid-sagittally through the IIIrd ventricle to produce homologous or mirror explant pairs which are developmentally and morphologically comparable (23). Each explant was placed on a collagen-coated coverslip and maintained in a Maximow slide assembly (1, 23) with one drop (50  $\mu\text{l}$ ) of a modified nutrient medium which was replenished 3–4 times a week. Modifications in the previously described (23) nutrient medium included: (1) horse serum (24%); (2) Eagle's minimal essential medium buffered with  $10^{-2}$  M HEPES (48%); (3) balanced salt solution (17%); (4) 10% glucose to achieve a final concentration of 900 mg/100 ml and (5) no chick embryo extract. Cultures were gassed with 5%  $\text{CO}_2$  in air after each feeding. Antibiotics were never used. Estradiol-17 $\beta$  ( $\text{E}_2$ ) (100 ng/ml) or testosterone (T) (1–5  $\mu\text{g}/\text{ml}$ ) (Sigma Chemical Company) was added in the nutrient medium at each feeding from explantation (birth) on to one explant of each homologous pair. Control cultures, the homologous explant half, received an equal volume of the diluent (0.5% bovine serum albumin in saline, BSA). These steroid dose levels, arbitrarily chosen as a starting point, were derived from in vivo experiments in the literature. Cultures were observed daily under bright-field microscopy and representative explants were fixed and stained as whole mounts for neurofibrils (Holmes' reduced silver nitrate). A total of 46 hypothalami, comprising 276 explant pairs, were studied for periods of up to 70 days.

Regardless of genetic sex, the addition of  $\text{E}_2$  or T at explantation results in striking morphological effects seen as early as the 4th day in vitro in both living cultures and silver-impregnated preparations. Steroid-sensitive cultures are characterized by an accelerated and progressively intense proliferation of neuronal processes or neurites from selected portions of specific hypothalamic levels. Consistently radiating out from the margins of some regions of the preoptic/anterior hypothalamus (figure 46.2a and b) and, to a somewhat lesser degree, from the mammillary levels as well



**Figure 46.1**

Diagram of the ventral surface of the newborn mouse brain showing the method of hypothalamic section to produce 6 regions. The procedure for obtaining homologous or mirror explant pairs, A + B, is shown in a diagram of a representative coronal section. The regions have been arbitrarily termed as shown on the basis of consistently observed and prominent topographic or morphologic features.

(figure 46.2c–f), this striking neuritic response which, at least in part, appears to be axonal in nature is further characterized by the formation of extraordinarily dense, cell-free plexuses of finely arborized fibers (figure 46.2c–f). The topographic distribution of this neuritic outgrowth frequently covers a very broad area, extending from the margin of the explant for distances at least 2.5–3 times that of the control (figure 46.2c and d). In addition, silver-impregnated structures characteristic of synaptic boutons and rings are frequently seen in contact with many fibers of the plexuses (figure 46.2f). The developmental precocity of steroid-sensitive regions is also manifested by enhanced neuronal maturation and myelinogenesis. The developmental parameters of counterparts from non-steroid-sensitive regions, on the other hand, are remarkably similar to one another and the topography of their neuritic outgrowth is virtually superimposable.

In order to investigate further this phenomenon, one explant of each homologous pair from 6 hypothalami (36 pairs) was exposed to nutrient medium whose serum component had been pretreated with antibodies to  $E_2$  in an attempt to reduce any endogenous estrogenic activity (2). The antibodies were raised in ovariectomized ewes to an estradiol-17-hemisuccinate–BSA antigen. Controls included normal serum, sheep serum and serum containing antibodies to BSA. These studies suggest that in striking contrast to the morphogenetic responses to exogenous steroid, the development of cultures deprived of  $E_2$  is retarded exactly opposite in manner and, most significantly, only in regions previously shown to be steroid-sensitive (figure 46.2g and h). Such cultures are characterized by: (1) a marked reduction and delay in neuritic out-

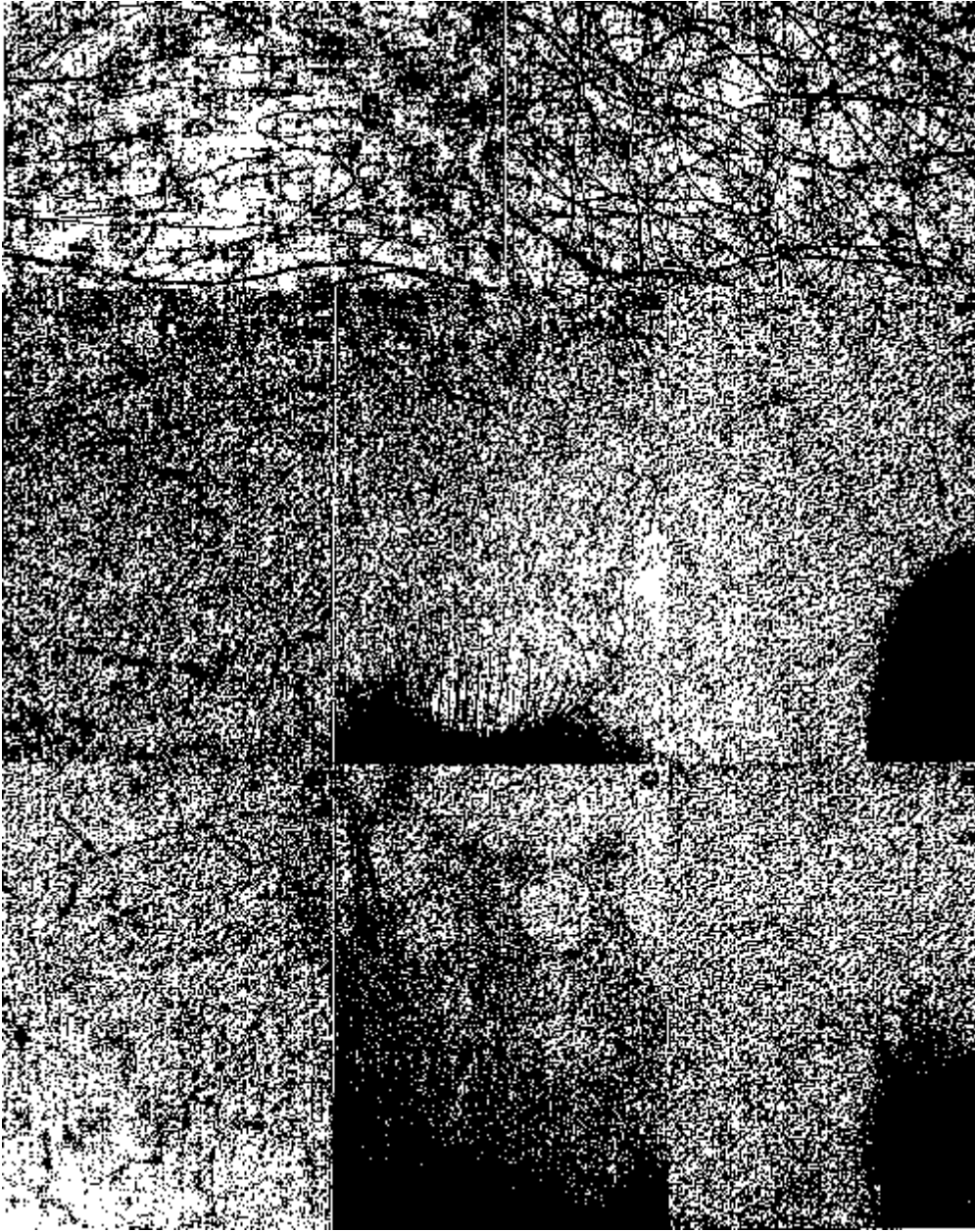
growth; (2) the proliferation of increased numbers of undifferentiated neuroblasts; and (3) significant retardation of neuronal maturation, including myelinogenesis.

What must be emphasized from these experiments is that while seemingly regionally specific, the neuritic (axonal) responses to the presence or absence of steroid should not be considered abnormal in themselves. They represent, rather, only alterations in the rate and magnitude of the basic (control) neuritic responses in this *in vitro* system.

Studies on the ontogeny of dendritic differentiation throughout the central nervous system (9, 10, 13, 18) have led to the general concept that the receptive parts of target neurons as well as dendritic geometry and synaptic patterns are induced by their afferent axonal input. Morest (13), furthermore, has observed *in situ* that dendritic differentiation generally follows that of the axon and that a close temporal relationship exists between the differentiation of dendrites and the appearance of afferent axonal endings in their immediate vicinity. Gottlieb and Cowan (6), moreover, have suggested that since the amount of postsynaptic space available appears to be strictly limited and relatively constant for each neuronal type, the repartition of synaptic sites between different groups of converging axons is determined competitively on a temporal basis. The spatial distribution of synapses, therefore, appears to reflect the differential growth rates of specified axons and the relative number of each axonal category present at the time of synaptogenesis. The site of termination of a particular afferent axon may also depend on the rate of maturation of its target neuron(s), since different regions of the receptive surface have been shown to differentiate at different times (22). Patterns of dendritic branching, spine density and synaptic organization, however, exhibit a considerable degree of plasticity, since environmental (3, 4, 7, 8, 11, 16, 21) and endocrine (19) factors acting postnatally can modify them.

The timing or rate of axonal development, therefore, shown to be critical in the establishment of neuronal interactions *in situ*, appears to be demonstrably influenced by the gonadal steroids *in vitro* as well. The very neuritic nature of this *in vitro* response, especially its temporal aspects, suggests that steroid-induced differences in axonal growth patterns may play a role in the neurogenesis of sexual differentiation. One might thus postulate that steroid-induced variations in both the timing and extent of afferent axonal development could so influence dendritic differentiation and synaptic distribution of target neurons as to result in fundamentally different, gender specific, patterns of neural organization.

Although the neuronal origin of the sexually differentiated axon terminals observed in the preoptic area



**Figure 46.2**

Photomicrographs of cultures of newborn mouse hypothalamus/preoptic area. *a* and *b*; *c* and *d*; *e* and *f*; and *g* and *h* are homologous (mirror) explant pairs (see text). *a* and *b*: living cultures of the preoptic area, 6 days in vitro ( $\times 125$ ). *a*: control. Numerous fine neurites (arrow) radiate outward singly and in organized bundles from the margin of the explant (E). *b*: testosterone  $1 \mu\text{g/ml}$ . The corresponding region (E) shows a marked increase in the neuritic outgrowth and the beginning of dense plexus formation. *c* and *d*: silver-impregnated cultures (Holmes') of the mammillary region, 19 days in vitro ( $\times 125$ ). *c*: control. Numerous silver-impregnated axons (arrow) course outward from the margin of the explant. *d*: estradiol  $100 \text{ ng/ml}$ . The neuritic outgrowth of the homologue with its extraordinarily dense plexus formation is striking. These axons extend  $1.6 \text{ mm}$  from the margin of the explant proper, a distance 2.75 times that of *c*. *e* and *f*: high power views of *c* and *d*,  $600 \mu\text{m}$  from the corresponding margins of the explants proper. Note in *f* the complexity of the axonal proliferation and the numerous synaptic structures (arrow) ( $\times 538$ ). *g* and *h*: living cultures of the preoptic area, 5 days in vitro ( $\times 125$ ). *g*: control. Neuritic bundles and individual neurites emerge from the margin of the explant. *h*: antibodies to estradiol. Note the marked delay in neuritic outgrowth in the homologue and the presence of numerous, small undifferentiated neuroblasts (arrow).

by Raisman and Field (17) is unknown, the nature and specificity of these in vitro observations, furthermore, support their suggestion that the axonal source may be derived from axons or axon collaterals intrinsic to this region. Organotypic cultures of central nervous tissue are derived from de- or non-afferented regional fragments and thus the subsequently regenerating or newly-developing axons can only arise from neurons originating within the explant proper; in this case the preoptic area.

These in vitro experiments suggest, in addition, that no pattern of sexual differentiation need necessarily be intrinsic to nervous tissue but that male and female patterns may *both* require active induction by steroid. Although high circulating levels of estrogens are present in the rodent of both sexes during the perinatal period, their functional activity may be significantly reduced because of binding to fetoneonatal extracellular binding protein (12). Exposure of the brain to such low extracellular levels of estrogen during the critical period, therefore, might induce, in the genetic female, a given pattern of neural organization. Intraneuronal aromatization of androgen to estrogen, on the other hand, could perhaps produce a more localized and concentrated estrogenic effect and the resulting stimulus to axonal development might thus induce a different, or male, pattern of differentiation. Ohno et al. (15) have independently proposed that neonatal imprinting might be a prerequisite for both masculinization and feminization of the brain by demonstrating the total absence of sexual behavior in mice with the testicular feminization mutation.

Preliminary studies, furthermore, suggest that the critical period may also exist in vitro, since inadequate or lack of exposure to testosterone for the first week in vitro results in homologous explant pairs whose development is entirely comparable.

Whether this response to steroid is a property of specific steroid-sensitive neurons or whether it represents, rather, the response of neurons in general at a specific developmental stage is unknown. These various aspects are currently under further study.

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Sexual dimorphism of the mammalian brain is thought to result from an irreversible organizational step during a “critical period” of development leading to a “male” or “female” wiring pattern in certain brain areas (see review by Maclusky and Naftolin [25]). Although this epigenetic action of sex steroids during neural differentiation is well established, little is known concerning cellular mechanisms. The in vitro studies of Toran-Allerand (36–39), Tixier-Vidal (6, 20), Stumpf et al. (35) and Uchibori and Kawashima (41, 42) have shown that sex steroids are capable of promoting survival and/or neurite growth and arborization in explants or dissociated cell cultures from embryonic and neonatal mouse hypothalamus, preoptic area and cerebral cortex. It has been suggested that such events may also occur in vivo leading to the sexually dimorphic synaptic organization found in specific brain regions (see reviews by Arnold and Gorski [2] and Toran-Allerand [39]). It is not clear, however, whether these effects are due to alterations in particular categories of neurons with specific transmitter phenotypes, or whether they are more generalized to estrogen receptor-containing regions in general.

Neurons which participate in neuroendocrine feedback mechanisms (e.g., catecholaminergic, 5-HT) are likely candidates for such effects, especially since sex differences in catecholamine and 5-HT metabolism begin to appear during the perinatal ‘critical’ period for the organizational effects of steroids (18, 43, 46). Moreover, Simerly et al. (31, 32) have reported different patterns of 5-HT fiber distribution in the sexually dimorphic nucleus of the medial preoptic area in adult males and females, which can be altered by treatment with perinatal sex steroids, and Giulian et al. (11) have reported that neonatal treatment with sex steroids cause different alterations in 5-HT levels in the male and female rat brain. Sex differences in the distribution of 5-HT fibers have also been reported in the rat spinal cord (17). The possible involvement of 5-HT in the development of sexual dimorphism has been suggested by Jarzab and Döhler (14), who demonstrated that male and female sexual behavior could be significantly inhibited by stimulation of 5-HT synthesis with L-

tryptophan treatment of neonatal rats, but not by inhibition of 5-HT synthesis by treatment with *p*-chlorophenylalanine (*p*CPA). Interestingly, effects of estrogen on 5-HT receptor (3) and sex differences in 5-HT receptor binding (7) have been reported, suggesting that the interaction of sex steroids and 5-HT receptors could be involved in the development of sexual dimorphisms related to this transmitter system.

Steroidal effects on other transmitter systems have also been reported, such as the dimorphic sprouting responses of sympathetic axons in the hippocampus of male and female rats in response to lesions of septal afferents (21, 26), and alterations in activity of tubero-infundibular dopaminergic neurons in response to neonatal androgen exposure (4). In addition, Dörner (5) has demonstrated changes in sexually dimorphic behavior in rats treated neonatally with either monoamine oxidase or acetylcholinesterase inhibitors.

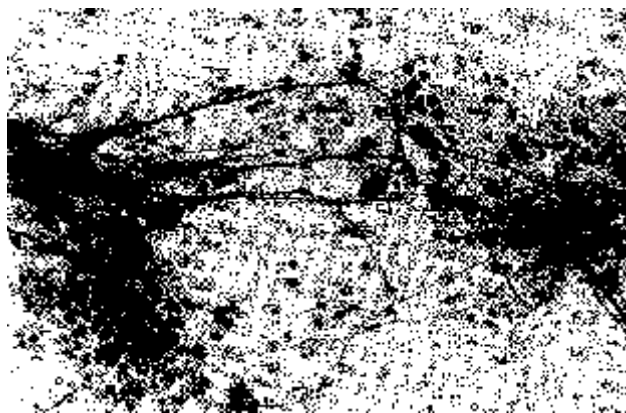
The present study was designed to investigate the possibility that during the fetal period, sex steroids may selectively influence the differentiation of monoamine neurons during prenatal development of the rat brain.

#### Experimental Procedures

Dissociated cultures of di-, mes- and metencephalon were prepared from embryonic day 14 (E14) Sprague–Dawley rats, using the mesencephalic and pontine flexures as landmarks, and immunocytochemical mapping of the location of TH- and 5-HT-IR neurons (34, 44) (Lauder, unpublished observations). The metencephalon was obtained by cuts at the pontine flexure and just caudal to the mesencephalic flexure. The mesencephalon was obtained by cuts just caudal and rostral to the mesencephalic flexure. The diencephalon was obtained from the remaining piece of tissue by removal of the cerebral hemispheres and the anterior forebrain (by a cut at the level of the optic chiasm). Cells were dissociated using trypsin and EDTA and plated on poly-L-lysine coated coverslips at an initial plating density of  $5 \times 10^5$  cells per well on 24-well plates, and cultured in Eagles Basal Medium (EBM) supplemented with 10% castrated horse serum for 6 days.

Concentrations of sex steroids were below the level of detection as tested by radioimmunoassay (Toran-Allerand, unpublished observations).  $17\beta$ -Estradiol (E), testosterone (T), estradiol plus testosterone (E + T), progesterone (P),  $5\alpha$ -dihydrotestosterone (DHT), or dexamethasone (DEX) were added daily to the medium in concentrations of  $10^{-8}$ – $10^{-10}$  M throughout the experiment. Stock solutions ( $10^{-2}$  M) were made by dissolving steroids in ethanol. No ethanol controls were done since the amount of ethanol added to the medium with steroids diluted from this stock to  $10^{-8}$ – $10^{-10}$  M was considered to be very small. Although possible effects of ethanol should not be ignored, since all steroid solutions contained ethanol, only certain steroids produced significant effects on neurite outgrowth (see Results). Thus we believe that the presence of this substance probably did not affect the outcome of these experiments. After 6 days in vitro (DIV), cultures were fixed in cold, 4% paraformaldehyde in 0.67 M phosphate buffer (pH 7.2) for 30 min and processed for immunocytochemistry using the avidin–biotin peroxidase (ABC) method (40), with characterized antisera to tyrosine hydroxylase (TH) or serotonin (5-HT) (15, 45).

To exclude the possibility of effects due to cell survival, the total number of immunoreactive (IR) cells per well were counted for each brain region (3 wells per group) and means compared between treatment groups. For mesencephalic cultures, lengths of neurites of IR cells were assessed with the help of an image processing system in 10 randomly chosen field areas of the cultures, defined by an ocular grid at  $\times 200$  magnification (grid area =  $0.16 \text{ mm}^2$ ). The image from each field area on the slides was drawn to a high resolution ( $640 \times 200$  pixel) monochrome computer screen with the aid of a camera lucida attachment. Only cell processes (not perikarya) were traced. To measure the lengths of processes in a given field, the computer counted the number of picture elements (pixels) that comprised the image. These numbers were multiplied by the number of microns per pixel (1.58) to yield micron lengths of processes per field. Measurements were made on 10 random fields per culture for each experiment (total of 3 experiments) and the total length of neurites calculated. In the case of the diencephalon and metencephalon, however, where fewer and more scattered IR neurons were present, which made the use of random fields less than optimal, neurites per neuron (20 randomly selected neurons per culture, total of 2–3 experiments) were measured. This type of analysis was not possible on mesencephalic cultures due to the high density of neurites. The measurements for each culture condition were pooled across experiments and statistical analyses performed on the individual measurements (i.e.,  $n = 30$ – $60$  measurements/culture



**Figure 47.1**

Tyrosine hydroxylase-immunoreactive (TH-IR) neurons in a culture from E14 mesencephalon treated with estradiol and testosterone for 6 days. Anti-TH immunocytochemistry and Toluidine Blue counterstain.  $\times 460$ .

condition). The values were ranked and statistical significance calculated using the Mann–Whitney test (33).

## Results

TH-IR cells were observed in cultures from all three brain regions, whereas 5-HT-IR cells were only present in metencephalic cultures. The survival of IR cells was not significantly affected by steroid hormone treatment in any of these cultures, as determined by counts of total TH or 5-HT-IR cells per culture. (Mesencephalic and diencephalic cultures contained 3600–4202 and 810–1096 TH-IR cells/well, respectively. Metencephalic cultures contained 1940–2120 5-HT-IR cells/well. No statistically significant differences were found between treatment groups for numbers of IR neurons/well.)

The effects of different steroids on neurite growth of TH- and 5-HT-IR neurons in dissociated cell cultures from E14 diencephalon (TH), mesencephalon (TH) and metencephalon (5-HT) are given in figures 47.2–47.4. Figure 47.1 illustrates a typical field of TH-IR neurons and neurites in a mesencephalic culture treated with  $17\beta$ -estradiol (E) and testosterone (T). Note the density of fibers, which makes measurement of neurites of individual neurons impossible. Both E and T significantly stimulated neurite growth in mesencephalic TH-IR neurons, and combined treatment with E + T produced a slightly greater stimulation than either sex steroid alone (figure 47.2). However, the other steroids investigated (progesterone, P;  $5\alpha$ -dihydrotestosterone, DHT; dexamethasone, DEX) had no statistically significant effects on the morphological differentiation of these neurons, although DEX produced a slight increase in neurite length. In contrast, effects of steroids in cultures of TH-IR neurons from the diencephalon

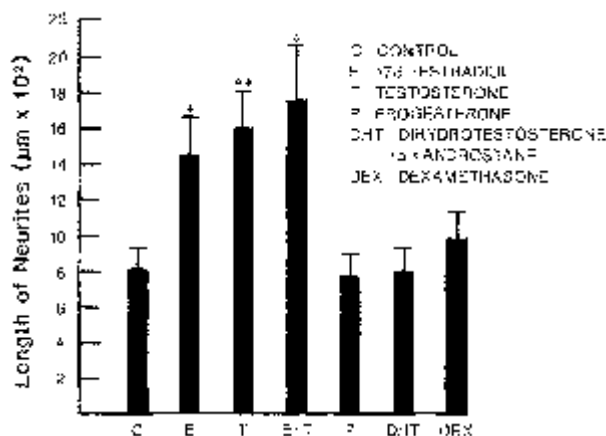


Figure 47.2

Effect of sex steroids on mesencephalic dopaminergic neurons after 6 DIV. Note the significant effect with testosterone, estradiol and testosterone plus estradiol treatments. Bars represent means of total length of neurites per field (10 random fields per culture, 3 experiments per treatment group,  $n = 30$  fields per treatment group). Error bars = S.E.M. \* $P < 0.05$ , \*\* $P < 0.01$  (Mann-Whitney test).

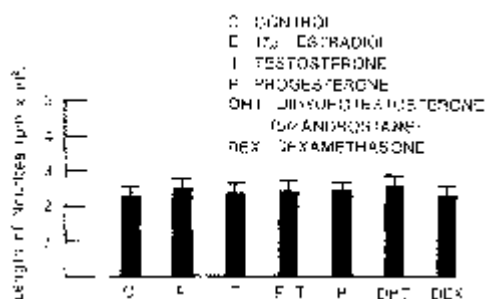


Figure 47.3

Effect of sex steroids on diencephalic dopaminergic neurons after 6 DIV. Bars represent means of total length of neurites per neuron (20 randomly selected neurons per culture, 3 experiments per treatment group,  $n = 60$  neurons per treatment group). Error bars = S.E.M. Mann-Whitney test showed no significant differences of treatment groups compared to controls.

(figure 47.3) and metencephalon (figure 47.4) were far less pronounced and no statistically significant differences were observed compared to controls.

## Discussion

These results suggest that sex steroids promote neuritic growth, but do not affect survival of cultured tyrosine hydroxylase-immunoreactive (TH-IR) neurons. This effect on neurite growth is most pronounced in TH-IR neurons in mesencephalic cultures. These cells are presumably dopaminergic (DA) based on their ability to take up [ $^3$ H]DA, which can be blocked by specific inhibitors (1) (Reisert et al., unpublished observations). In contrast, no significant effects of these steroids were observed in cultures of TH-IR neurons from the diencephalon or 5-HT-IR neurons from the metencepha-

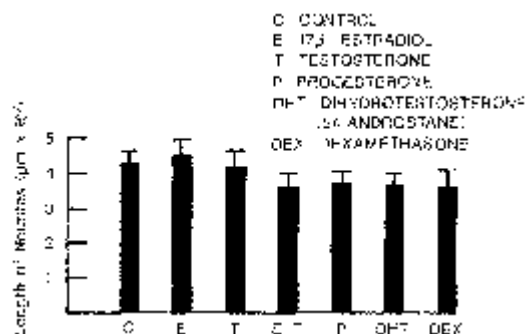


Figure 47.4

Effect of sex steroids on metencephalic serotonergic neurons after 6 DIV. Bars represent means of total length of neurites per neuron (20 randomly selected neurons per culture, 2 experiments per treatment group,  $n = 40$  neurons per treatment group). Error bars = S.E.M. Mann-Whitney test showed no significant differences of treatment groups compared to controls.

lon. Thus, it appears that there may be a specific effect of estradiol and testosterone on neurite growth of mesencephalic DA neurons in cultures prepared from the E14 rat brain.

The physiologic significance of these findings is underlined by the low concentrations of hormones used in our experiments ( $10^{-8}$ ,  $10^{-10}$  M). These results might not have been detectable if castrated horse serum had not been used (which has levels of sex steroids below the level of detection by radioimmunoassay; Toran-Allerand, unpublished observations). Friedman et al. (8) reported that growth of embryonic DA neurons from mouse substantia nigra and basomedial hypothalamus was unaffected by culturing in medium containing serum from ovariectomized/adrenalectomized rats. We also obtained healthy cultures using castrated horse serum, but their growth could be enhanced by the addition of sex steroids.

The fact that treatment with estradiol (E) or testosterone (T) enhanced neurite elongation to about the same extent may be due to the conversion of E to T by aromatase, which has been reported to occur in mouse neural cultures (39) and neonatal rat brain (23). The effects of E and T on morphogenetic differentiation of mesencephalic TH-IR neurons could have been mediated by steroid receptors, since Toran-Allerand has found nuclear concentration of labeled estradiol in explant cultures (37, 39). However, binding of sex steroids by adult mesencephalic DA neurons has not been observed in vivo (12, 13), although steroid receptors have been found in hypothalamic DA neurons (29).

The development of estrogen receptors in mouse hypothalamus begins at about E14–15 (9, 10, 16), which corresponds to E15–16 in the rat. Thus it is possible that at the time of culture, few DA neurons from this region have estrogen receptors. On the other hand,

developing mesencephalic DA neurons might transiently express receptors for estrogen before such receptors are present on hypothalamic DA neurons. In support of this idea is the finding of transient expression of estrogen receptors in perinatal cerebral cortex by assay (9, 22, 24) and autoradiography (10, 30). However, such receptors have not been found in mesencephalic DA neurons at any stage of embryonic development in vivo, although they have been demonstrated in 5-HT neurons of the dorsal raphe nucleus at E16 (35). This could be due to insufficient time for exposure to autoradiographic material, as in previous experiments in cortex, where estrogen receptors were not found until very long times were used (10). Alternatively, it is possible that the observed in vitro effects of sex steroids are indirect, due to the action of these hormones on other cells in the culture system (e.g., glia) which then influence the differentiation of mesencephalic DA neurons (see Lauder and McCarthy [19] and Toran-Allerand [39]).

Thyroid hormones have also been found to exert differential effects on mesencephalic and hypothalamic DA neurons in vitro. For example, triiodothyronine ( $T_3$ ) was shown to enhance neuritic growth and increase cell body size in hypothalamic cultures from the E16 mouse brain (27). In mesencephalic cultures (28), however, only cell body size was increased, with no effect on neurite growth. No effects on neuronal survival were seen with either culture condition.

The results of this study indicate that sex steroids may influence the differentiation of mesencephalic dopaminergic neurons during the fetal period. This could be significant in terms of development of sexual dimorphism, either within this group of monoamine neurons or in brain regions to which they project.

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The basal forebrain is a region that, in the human, is important for learning, memory, and other cognitive functions and in which the cholinergic neurons are affected early in Alzheimer disease and age-related cognitive impairment (1, 2). Developing and adult basal forebrain neurons are targets not only of the family of neurotrophins—nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin 3 (NT-3)—but of the gonadal steroid estrogen as well. Basal forebrain cholinergic neurons of the developing rodent and primate have been shown to express the mRNA (3, 4) or encoded protein (5, 6) for the low-affinity form of the NGF receptor, which binds the neurotrophins (7, 8). Overlapping basal forebrain regions have also been shown to exhibit estrogen-receptor mRNA, estrogen binding sites, and high levels of the estrogen synthesizing enzyme cytochrome P450-dependent aromatase (for refs. see ref. 9), although the neurotransmitter phenotype(s) of these neurons has not been reported. A critical question concerning the developmental effects of estrogen in the brain is whether these actions are exerted directly. Alternatively, the steroid may potentiate synthesis of endogenous growth and trophic factors and their receptors (10), as in extra-neural targets (11, 12), or it may interact with them in a stimulatory or inhibitory fashion to promote the growth and differentiation of specific neuromodulatory or neurotransmitter systems by autocrine or paracrine mechanisms, which may lead to shifts in the developmental patterns of resulting neural networks.

To test the hypothesis that estrogen and the neurotrophins may act on the same basal forebrain neurons, we combined autoradiography with  $^{125}\text{I}$ -labeled estrogen ( $^{125}\text{I}$ -estrogen) and nonisotopic in situ hybridization histochemistry or immunohistochemistry to identify the mRNA and encoded protein for the low-affinity (p75<sup>NGFR</sup>) NGF receptor and for choline acetyltransferase (ChAT; acetyl-CoA: choline *O*-acetyltransferase, EC 2.3.1.6), the acetylcholine-synthesizing enzyme, a marker of cholinergic neurons. We report here the colocalization of estrogen binding sites with the low-affinity NGF receptor p75<sup>NGFR</sup> mRNA and immunoreactive protein in neuronal sub-

sets of the rodent medial septum, nuclei of the diagonal band of Broca, and in the continuum of neurons of the ventral pallidum, substantia innominata, and nucleus basalis of Meynert. Moreover, we also provide definitive evidence that cholinergic basal forebrain neurons bind estrogen as well.

## Materials and Methods

### Animals

Postnatal female Sprague–Dawley rats (Charles River Breeding Laboratories) 10 and 12 days old ( $n = 15$ ) and adult female mice (RIII) from our breeding colony, ovariectomized under methoxyflurane (Metofane) for 1 week before use ( $n = 5$ ), were injected s.c. or i.v. in the jugular [ $1 \mu\text{Ci}$  per g of body weight ( $1 \text{ Ci} = 37 \text{ GBq}$ );  $\approx 0.2 \text{ ng}$  per g of body weight] with 11 $\beta$ -methoxy[16 $\alpha$ - $^{125}\text{I}$ ]iodoestradiol ([ $^{125}\text{I}$ ]MIE<sub>2</sub>) synthesized by R.B.H. as described (13) (100  $\mu\text{l}$ ; 50% propylene glycol/50% saline) and killed 1 h later by transcardial perfusion under deep ether anesthesia with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.6) containing 2.5% (vol/vol) dimethyl sulfoxide (DMSO) and 0.1% of the RNase inhibitor diethylpyrocarbonate. Specific binding of this iodinated estrogen is completely abolished by concurrent exposure to 100-fold molar excess of the unlabeled nonsteroidal estrogen diethylstilbestrol (14). Brains were postfixed for 2 h at 4°C in the same fixative, without DMSO, and then equilibrated overnight at 4°C in 15% buffered sucrose before embedding in M-1 embedding matrix (Lipshaw Manufacturing, Detroit) and freezing on dry ice/acetone as described (14).

### Steroid Autoradiography

Steroid autoradiography was carried out first. Coronal sections (10–12  $\mu\text{m}$ ) through the basal forebrain region were cut on a cryostat and thawmounted onto NTB-3 (Kodak) emulsion-coated slides and processed for steroid autoradiography combined with either immunohistochemistry (14) or nonisotopic (digoxigenin) in situ hybridization histochemistry as described (9). After photographic development, the autoradiograms were

processed for immunohistochemistry with antibodies raised against either p75<sup>NGFR</sup> [monoclonal antibody IgG 192 (15); 1:25; gift of Eugene M. Johnson, Jr., Washington University, St. Louis] or ChAT (rabbit polyclonal antibody; 1:500; Chemicon) by means of the avidin-biotin-peroxidase complex method with 3,3'-diaminobenzidine as the chromagen (Vectastain ABC Elite kit, Vector Laboratories). Both antibodies have been extensively characterized and both antigens survive autoradiographic development [D170, 2.5% sodium sulfite/0.1% potassium bromide/0.45% 2,4-diaminophenol dihydrochloride (Kodak), pH  $\approx$  7 (9, 14)].

### In Situ Hybridization Histochemistry

Nonisotopic (digoxigenin) in situ hybridization histochemistry was carried out after steroid autoradiography for p75<sup>NGFR</sup> and ChAT mRNA as described (9). Briefly, digoxigenin-labeled oligonucleotides were synthesized as described (16, 17). The oligonucleotides were 3'-end-labeled with digoxigenin-labeled deoxyuridine triphosphate (dUTP) (Boehringer Mannheim) by terminal deoxynucleotidyl transferase (BRL). The developed autoradiograms were hybridized overnight at 35°C (NTB-3 emulsion melts off the slide at temperatures  $>37^{\circ}\text{C}$ ) with 50 ng of probe per ml as well as with 2 pmol of a random composition, unlabeled, 36-base oligonucleotide per ml containing  $>10^{20}$  sequence combinations (DuPont; NEP 550) added to decrease nonspecific hybridization. After hybridization to the target cDNA, the hybrids were detected by enzyme-linked immunohistochemistry with anti-digoxigenin antibodies conjugated to alkaline phosphatase (Fab fragment, 1:500; 48 hr; 4°C) (Boehringer Mannheim) and an enzymecatalyzed, blue-color reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt (Boehringer Mannheim). After washing at 35°C and at relatively high stringency, sections were cleared in Histo-Clear (National Diagnostics, Manville, NJ) (to avoid fading of the color product) and mounted in Permount.

### p75<sup>NGFR</sup> mRNA

Because monoclonal antibody IgG 192 recognizes the low-affinity NGF receptor of the rat only (15), the distribution of immunoreactive p75<sup>NGFR</sup> could be studied only in autoradiograms of developing rats. To minimize the amounts of [<sup>125</sup>I]MIE<sub>2</sub> needed and to address this question in the adult rodent, nonisotopic in situ hybridization histochemistry was carried out for p75<sup>NGFR</sup> mRNA in the much smaller adult mouse, as well as in developing rats. The p75<sup>NGFR</sup> probe was a 46-base synthetic oligonucleotide sequence from the membrane-spanning region of chicken low-affinity NGF receptor cDNA (18), which has been extensively

characterized and which shares extensive homology with the low-affinity rat NGF receptor.

### ChAT mRNA

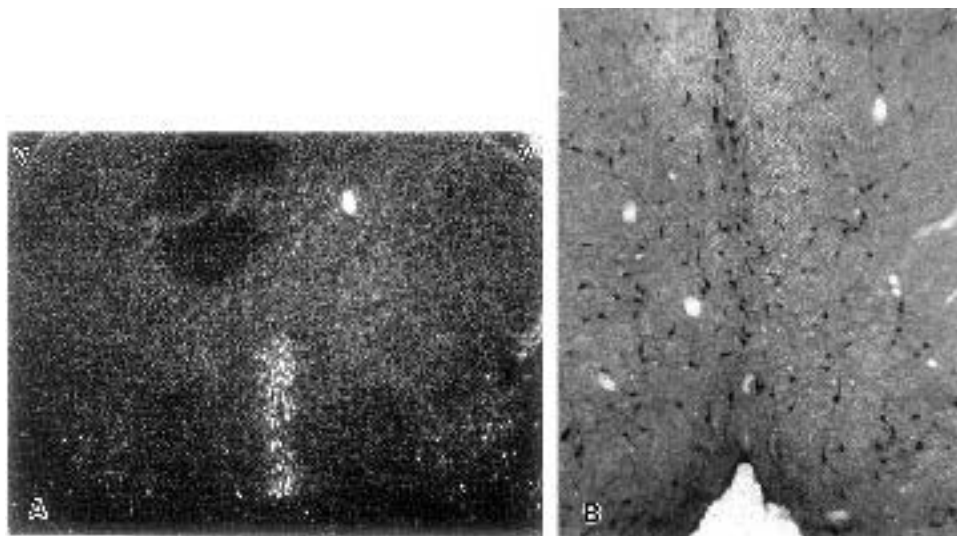
Two synthetic oligonucleotides, 39 and 48 bases, were synthesized from opposite ends of the cDNA sequence that encodes active rat ChAT (19). The first corresponds to bases 202–243, which is comparable to that used in molecular biological studies of the enzyme (20). The second spans bases 1120–1168, which corresponds to the porcine ChAT sequence used in enzyme induction studies (3). The p75<sup>NGFR</sup> and two ChAT probes have no significant homology with any of the known nucleotide sequences in the GenBank/EMBL data bases.

Controls for the specificity of p75<sup>NGFR</sup> and ChAT mRNA detection included, as described (9, 17): (i) the demonstration on Northern blot analysis that signal detection by each probe hybridized only to a species of RNA of a size corresponding to that of the mRNA encoding the appropriate protein; (ii) incubation with noncomplementary (sense) digoxigeninlabeled probes; (iii) pretreatment with excess unlabeled 90-base oligonucleotides synthesized against sequences that overlapped the experimental probes; and (iv) analyses of thermal stability, which showed a close match ( $\approx 1^{\circ}\text{C}$ ) between theoretical and experimentally calculated melting temperatures, indicating hybridization to a single species of mRNA containing the expected sequence. Neurons expressing p75<sup>NGFR</sup> or ChAT immunoreactivity or the blue cytoplasmic hybridization signal for their mRNA were characterized with respect to the presence or absence of discrete concentrations of silver grains in the emulsion underlying cell nuclei, indicating binding of the iodinated estrogenic ligand. [<sup>125</sup>I]MIE<sub>2</sub> binding was considered specific, using a  $5\times$  background labeling criterion, where the probability of false labeling is  $<0.001$  (21).

## Results

### Distribution of Estrogen and Low-Affinity NGF Receptors

Estrogen receptor mRNA expression and estrogen binding sites (figure 48.1A) in the basal forebrain are distributed in a pattern that clearly overlaps the expression of p75<sup>NGFR</sup> mRNA and its encoded immunoreactive protein (figure 48.1B). As will be shown elsewhere, virtually all neurons of the medial septum/diagonal band coexpress estrogen receptor and p75<sup>NGFR</sup> mRNA by double-label (<sup>35</sup>S/digoxigenin) in situ hybridization (R.C.M., F.S., and C.D.T.-A., unpublished data). Analysis of autoradiograms of the developing rat medial septum, nuclei of the horizontal and vertical limbs of the diagonal band, the ventral pallidum, substantia innominata, and the nucleus basalis that had been



**Figure 48.1**

Spatial distribution of estrogen binding sites and low-affinity NGF receptor immunoreactivity clearly overlaps in the P10 rat medial septum and nuclei of the diagonal band. (A) Dark-field autoradiogram of [<sup>125</sup>I]MIE<sub>2</sub>-concentrating neurons in the vertical nucleus of the diagonal band (V, ventricle). (B) NGF receptor immunoreactivity in the medial septum and nuclei of the diagonal band. (A,  $\times 15$ ; B,  $\times 5$ .)

reacted with antibodies to the p75<sup>NGFR</sup> protein revealed numerous estrogen-concentrating neurons colocalizing p75<sup>NGFR</sup> immunoreactivity. Neurons in these regions, however, were heterogeneous with respect to colocalization of estrogen and NGF receptors. Many neurons that exhibited p75<sup>NGFR</sup> immunoreactivity colocalized estrogen binding sites as well (figure 48.2A and B). A smaller number of NGF receptor-positive neurons, in contrast, were estrogen receptor negative but were found admixed with those colocalizing NGF and estrogen receptors (figure 48.2C). Some NGF receptor-positive but estrogen receptor-negative neurons were also interspersed with still other neurons that exhibited either estrogen binding only (figure 48.2D) or neither phenotype (data not shown).

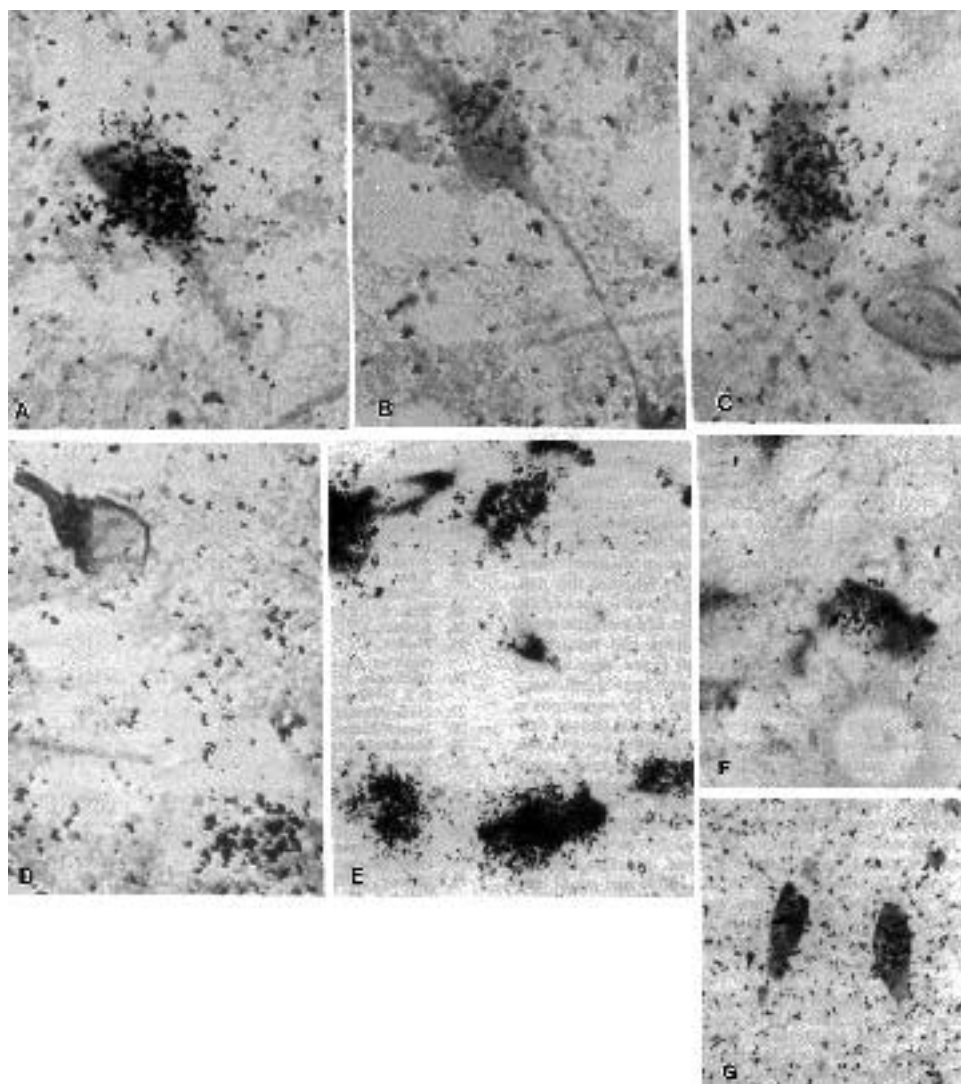
To address the potential for p75<sup>NGFR</sup> expression in the adult rodent, steroid autoradiography was combined with nonisotopic in situ hybridization histochemistry for p75<sup>NGFR</sup> mRNA expression in the smaller, adult mouse as well as in developing rats. Colocalization of the blue/purple cytoplasmic hybridization signal of p75<sup>NGFR</sup> mRNA and concentration of silver grains underlying cell nuclei were seen in estrogen target neurons of the same adult murine and developing rat basal forebrain regions where NGF receptor immunoreactivity was found in the developing rat (figure 48.2E).

To identify the phenotype of these estrogen-binding neurons, the autoradiograms were also processed for ChAT mRNA or its immunoreactive protein. Our studies demonstrate that many estrogen-binding neurons of the medial septum, nuclei of the diagonal band, substantia innominata and ventral pallidum, like the p75<sup>NGFR</sup>-containing cells they may represent, at least

in part, are cholinergic and coexpress ChAT mRNA (figure 48.2F) or ChAT immunoreactivity (figure 48.2G). What other neurotransmitter phenotype(s) the estrogen targets may also represent remains to be determined.

## Discussion

Our findings demonstrate that estrogen target neurons not only have the potential for p75<sup>NGFR</sup> and ChAT expression (by localizing the mRNA) but do, in fact, synthesize the encoded protein (by identifying immunoreactive NGF receptors and ChAT). Thus, basal forebrain cholinergic neurons may be influenced not only by the neurotrophins but by estrogen as well. Both ChAT and acetylcholinesterase, which normally increase in the basal forebrain in response to NGF during development (4) and in the adult (3), have also been shown to be estrogen responsive and to exhibit a sex difference in their activity and regulation, with the location and direction of change dependent on sex (22). Recent studies have shown that NGF promotes cholinergic neuron survival in the medial septum/diagonal band, after partial or complete transections of the fimbria-fornix, the septo-hippocampal pathway (23, 24), and in cognitively impaired aged rats (25). Whether or not estrogen exerts a modifying influence on this process is unknown. However, localization of aromatase, an enzyme that converts testosterone to the active estrogenic metabolite estradiol to these regions throughout life (26) provides the basal forebrain with a potential endogenous source of estrogen and thus a sex difference in estrogen availability.



**Figure 48.2**

Colocalization of estrogen binding sites with low-affinity NGF receptors and ChAT (mRNA and the encoded immunoreactive protein) in neurons of the developing postnatal days 10 and 12 (P10 and P12) female rat or adult female mouse basal forebrain. These results were obtained by combining [ $^{125}$ I]MIE<sub>2</sub> autoradiography (discrete concentration of silver grains in the emulsion underlying neuronal nuclei) with either immunocytochemistry (brown cytoplasmic reaction product) or nonisotopic (digoxigenin) in situ hybridization histochemistry (blue cytoplasmic hybridization signal). (A and B) Colocalization of estrogen and NGF receptors in P12 neurons of the medial septum/diagonal band (A) and basal nucleus of Meynert (B). (C) NGF receptor-positive/estrogen receptor-positive P12 neuron adjacent to an NGF receptor-positive/estrogen receptor-negative neuron. (D) NGF receptor-positive/estrogen receptor-negative neuron (*top left*) in the same field as a NGF receptor-negative/estrogen receptor-positive neuron (*bottom right*). (E) Colocalization of estrogen binding sites and NGF receptor mRNA in neurons of the horizontal nucleus of the diagonal band of the adult female mouse. (F and G) Colocalization of estrogen receptors with ChAT mRNA (F) or its encoded immunoreactive protein product (G) in the P10 rat. (A–D,  $\times 340$ ; E–G,  $\times 270$ .)

Gonadal steroid enhancement of neurite growth and of neuronal survival has been demonstrated in vitro and in vivo not only during development (ref. 27; for refs. see ref. 28), but in steroid receptor-containing regions (including the septum) of the deafferented, axotomized, or steroid-deprived adult central nervous system (CNS) as well (29–31). These findings suggest that the potential for steroid responsiveness may also extend into adulthood, but only after insult to the CNS. Such a pattern is reminiscent of the injury-induced, up-regulation of p75<sup>NGFR</sup> expression and the return of sensitivity to NGF not only in the basal forebrain of the adult rat (3) but also in the adult striatum (32), normally a target of NGF only during development (33).

Colocalization of the estrogen and NGF receptor systems implies that their ligands may each act on the same neuron, perhaps synergistically. Such interactions may regulate (enhance or suppress) by autocrine and/or paracrine actions the expression of specific genes or gene networks to influence neuronal survival, differentiation, regeneration, and plasticity. Conversely, loss of the stimulus mediated through either receptor may result in neuronal atrophy or even cell death. Structural and functional analyses of some of the genes regulated by estrogen reveal a common 13-base-pair palindromic sequence, [5'-GGTCANNNTGACC-3'] (34), which, while often imperfect, is sufficient to mediate hormonal induction of transcription. The possibility that coexpression of the estrogen and NGF receptors may be of biological significance is heightened by our preliminary findings (R.C.M., F.S., and C.D.T.-A., unpublished data), which suggest that the human low-affinity p75<sup>NGFR</sup> gene contains an estrogen response element (ERE)-like consensus sequence within its promoter region (bases -341 through -352) (35). This sequence includes the exact spacing of 3 base pairs between the two pentameric halves of the palindrome, which is essential for estrogen receptor action (34). Since the rat low-affinity NGF receptor gene has not been cloned, particularly in the 5' and promoter regions, the presence of ERE-like sequences is unknown. However, since the extracellular domain of p75<sup>NGFR</sup> is conserved throughout evolution (36), an ERE may also be present in the rodent. The functionality of this ERE remains to be evaluated.

It should be emphasized that NGF may not be the sole ligand acting on neurons where estrogen receptors and p75<sup>NGFR</sup> colocalize. The low-affinity state of the NGF receptor, which is the form recognized by monoclonal antibody IgG 192, has also been shown to bind with low affinity to other neurotrophins such as BDNF and NT-3 (7, 8, 37). Thus, basal forebrain neurons coexpressing estrogen binding and immunoreactivity for the low-affinity NGF receptor may reflect not only

potential NGF, but BDNF and possibly NT-3 responsiveness as well. BDNF, like NGF, has been shown to increase survival and acetylcholinesterase activity of septal cholinergic neurons in culture (38). Binding of the neurotrophins by p75<sup>NGFR</sup>, however, does not alone induce cellular responses. Neurotrophin signal transduction appears to require association of p75<sup>NGFR</sup> with members of the tyrosine kinase receptor family of protooncogene products—*trk* [tropomyosin receptor kinase (39)] and the structurally related genes *trkB* (40) and *trkC* (41). Thus, some forebrain regions known to express p75<sup>NGFR</sup> have also been shown to localize *trk* and *trkB* mRNA (40, 42, 43). As will be shown elsewhere, recent evidence from our laboratory by double-label in situ hybridization indicates that the estrogen receptor mRNA-containing neurons of the basal forebrain also colocalize the mRNAs for *trk* and *trkB* (R.C.M., F.S., and C.D.T.-A., unpublished data). Colocalization of estrogen receptor mRNA with *trk*, *trkB*, and p75<sup>NGFR</sup> mRNAs in the basal forebrain increases the importance of the findings presented here by suggesting that those estrogen target neurons that coexpress p75<sup>NGFR</sup> mRNA and protein have the potential for expressing functional neurotrophin receptors and thus for being regulated by the neurotrophins. Moreover, these findings also imply the potential for reciprocal regulation of the estrogen and neurotrophin receptor systems by their ligands by documenting the substrate for estrogen regulation of the neurotrophin receptors as well.

Whether or not all the estrogen receptor-containing neurons that express ChAT or p75<sup>NGFR</sup> represent the same subset is unknown at this time. Recent studies have documented that the majority of the NGF receptor immunoreactive neurons in the rodent (44) medial septum, diagonal band, and nucleus basalis are cholinergic, as determined by colocalization of markers such as ChAT. However, although most of the estrogen receptor-containing cholinergic neurons may be targets of NGF or of the other neurotrophins, not all of the estrogen- and NGF-receptor-containing neurons are likely to be cholinergic. Preliminary observations (C.D.T.-A., R.C.M., T.J.B., R.B.H., and N.J.M., unpublished data) suggest that some of the <sup>125</sup>I-estrogen-concentrating neurons of the medial septum/diagonal band may be  $\gamma$ -aminobutyric acid (GABA)-ergic in that they also contain immunoreactive parvalbumin, which has been shown to colocalize with GABA in neurons of the medial septum/diagonal band, hippocampus, and cerebral cortex (45). Whether this neuronal population is p75<sup>NGFR</sup> containing as well is not known.

If interactions between neurotrophins and estrogen are of fundamental importance for development and survival of target basal forebrain neurons in both sexes,

then clinical conditions that are associated with gonadal steroid deficiency could contribute to the atrophy or death of these neurons in both sexes. Some studies suggest that gonadal steroid deficiency may have a profound impact on cognitive function (46, 47). Similar considerations apply to the possible involvement of gonadal steroids in age-associated neurodegenerative disease states, where a declining stimulus from circulating estrogen or aromatizable androgen levels might make estrogen target neurons in the brain less resistant to other age- or disease-related processes. In both sexes, therefore, the natural decline in gonadal steroid levels in old age could contribute to the loss of neuronal systems vital to cognitive function, whether this occurs to the extreme observed in Alzheimer disease or follows the less traumatic path associated with normal aging.

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It is well established that the steroid hormone estradiol exerts profound effects on reproductive behavior in female rats through its action on hypothalamic nerve cells (Pfaff, '80). The minimal temporal requirements of exposure to estradiol ( $E_2$ ) for activation of progesterone-facilitated lordosis at 24 hours have been determined to be two exposures to  $E_2$  of 1 hour each separated by 4–13 hours (Parsons et al., '82a,b). Accumulated evidence from steroid hormone autoradiographic (Pfaff and Keiner, '73),  $E_2$  implant (Barfield and Chen, '77; Davis et al., '82), lesion (Pfaff and Sakuma, '79; Mathews and Edwards, '77), ultrastructural (Cohen and Pfaff, '81; Carrer and Aoki, '82; Cohen et al., '84), and electrophysiological (Moss and Foreman, '76) studies indicates that an important anatomical site of estrogenic regulation of rat female reproductive behavior is the ventromedial nucleus (VMN) of the hypothalamus and, especially, the neurons within the ventrolateral portion of the VMN.

A general mechanism of action of steroid hormones involves binding of the steroid hormone to its receptor, activation of the steroid-receptor complex, and subsequent binding of the activated steroid receptor to a nuclear acceptor site (Jensen et al., '82), which is currently thought to be a DNA-nonhistone chromosomal protein complex (Spelsberg et al., '83). These initial events in hormone action result in altered transcriptional activity (Gorski and Nicolette, '63; Hamilton et al., '65) and, ultimately, marked increases in the synthesis of steroid-specific proteins (O'Malley and Means, '74). Temporally, the changes in RNA synthesis have been found to occur within minutes (Means and Hamilton, '66) and subsequent induction of new proteins within hours (O'Malley and Means, '74) after hormone administration. While the effects of hormones on RNA and protein synthesis remain to be elucidated and are least understood in neuronal tissue, studies using RNA and protein inhibitors suggest that the time course of estrogenic action on nerve cells is in keeping with non-neural steroid hormone target tissues (Terkel et al., '73; Quadagno and Ho, '75). When the protein synthesis inhibitor anisomycin was given between two 1-hour segments of  $E_2$  administration, behavioral defi-

cits at 24 hours occurred (Parsons et al., '82b), suggesting that some of the proteins required for lordosis were synthesized within a few hours of exposure to the hormone.

The morphology of active gene transcription has been established through ultrastructural, high-resolution autoradiographic, and cytochemical studies, which have recently been thoroughly reviewed (Puvion-Dutilleul, '83; Knowler, '83). Pertinent morphological correlates of altered RNA synthesis and processing include changes in nucleolar and nuclear size (Busch and Smetana, '70; Merriam, '69), conformational changes in nucleolus-associated chromatin (Jones, '83), and distributive changes in the ribonucleoprotein-containing components of the nucleolus and the nucleoplasm (Fakan and Puvion, '80). Chromatin decondensation in uterine cell nuclei occurring within an hour of exposure to estrogen has been shown to be prevented by the RNA inhibitor actinomycin D (Vic et al., '80). In VMN neurons, chronic  $E_2$  treatment produces structural changes in the nucleoli (Cohen et al., '84).

To study early changes following estrogenic action on VMN neurons, ultrastructural and morphometric analyses of neurons in the ventrolateral portion of the VMN nucleus in ovariectomized rats after either a short exposure to  $E_2$  (2 hours) or a discontinuous treatment with  $E_2$  (2 hours on/7 hours off/2 hours on) were undertaken in this investigation. Since the initial components of estrogenic action on target tissue appear to involve nuclear-related events, we chose to focus our analyses principally on the cell nucleus.

## Materials and Methods

### Animals and Hormone Administration

Twelve female Sprague-Dawley rats weighing between 200 and 225 g were obtained from Charles River and ovariectomized (OVX) under methoxyflurane anesthesia 5–7 days prior to the start of experimentation. Crystalline estradiol ( $E_2$ ) was packed into 5-mm lengths of Silastic tubing, incubated in 0.1% phosphate-buffered saline for 24 hours prior to experimental use, and implanted subcutaneously under

methoxyflurane anesthesia according to the following time course. In the first group (2 hours of  $E_2$ ), three rats received the  $E_2$  silastic implants for 2 hours, with three OVX sham-operated rats serving as controls. In the second group (2/7/2 hours of  $E_2$ ), three OVX rats initially received the  $E_2$  silastic implants for 2 hours. The implants were then removed for 7 hours and reimplanted for an additional 2 hours. Three OVX rats were sham-operated under the same time course and served as controls.

### Tissue Preparation

At the end of the appropriate hormone administration time, each animal was anesthetized by intraperitoneal injection of Nembutal and fixed via intracardiac perfusion with a dilute fixative consisting of 1% paraformaldehyde-1.25% glutaraldehyde in 0.2 M sodium cacodylate buffer at pH 7.3 followed by a concentrated fixative consisting of 4% paraformaldehyde-5% glutaraldehyde in the same buffer (Peters, '70). After perfusion, the animals were left undisturbed for a minimum of 1 hour to prevent postmortem artifacts (Cammermeyer, '60). The brains were then removed and placed in concentrated fixative overnight at room temperature.

Following gross dissection of the hypothalamus using external landmarks, an Oxford vibratome was employed to isolate 200- $\mu$ m-thick sections containing the ventromedial nucleus (VMN), which was identified by dilute methylene blue staining. From these sections, the ventrolateral portions of the VMN were dissected and placed in separate vials containing 0.2 M sodium cacodylate buffer (pH 7.3). The advantage of this method was that small pieces of tissue containing only the ventrolateral portion of VMN could be obtained. These tissue pieces were washed again in buffer, post-fixed in 1%  $OsO_4$ , rinsed in buffer, dehydrated in successive changes of acetone, and finally embedded in Epon. The blocks were polymerized at 65°C for 36 hours.

### Electron Microscopy

Thin, 70-nm sections were cut using a Porter-Blum, MT-2 ultramicrotome and double stained with saturated alcoholic uranyl acetate for 5 minutes followed by Reynold's lead citrate stain ('63) for 3 minutes. The stained sections were examined with a JEOL JEM 100CX transmission electron microscope and recorded on Kodak electron image film. Electron micrographs of each neuron containing both nucleus and nucleolus in a given section cut from blocks selected randomly were systematically collected until a total of 25 neurons per rat was attained. Each neuron was photographed at a magnification of 5,000 $\times$  and the nucleolus within each nucleus photographed at 20,000 $\times$ . Thus, 300 neu-

rons were collected for this portion of the investigation, for a total of 600 electron micrographs. Subsequent ultrastructural analysis was accomplished independently by two investigators. One investigator examined the electron micrographs after partitioning them into experimental and control groups while the other investigator examined the electron micrographs, which were categorized according to groups, without prior knowledge of the group to which they belonged. In this manner, ultrastructural changes following  $E_2$  administration were determined by cross-comparison both within and between nuclear groups, and independently confirmed by "blind" analysis.

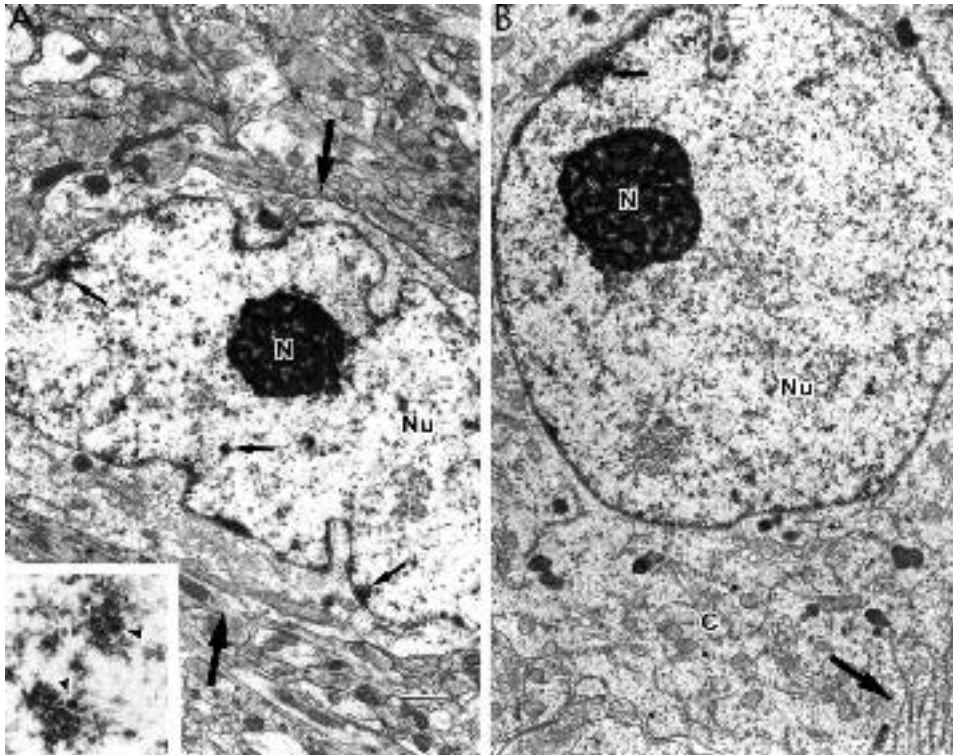
Cellular parameters selected for this initial analysis included the following: nuclear shape, nuclear size, character of the nucleoplasm, nucleolar morphology and size, amount of nucleolus-associated chromatin, and somal size; and the presence or absence of (1) a large heterochromatin clump along the nuclear envelope, (2) nuclear envelope-RER connections, (3) stacked RER, and (4) nuclear envelope invaginations.

### Morphometric Procedures

From 1- $\mu$ m sections cut from Epon-embedded blocks on a Porter-Blum, MT-2 ultramicrotome and stained with toluidine blue, nuclei, nucleoli, and somata were traced at 1,500 $\times$  with a Zeiss microscope with a camera lucida drawing attachment. All VMN neurons possessing nucleoli and clearly defined nuclear envelopes within a section were traced. One section from each of three tissue blocks was used, with a total of three sections taken from three different, randomly selected areas of the ventrolateral portion of VMN. Fifty nuclei, nucleoli, and somata per rat were collected, for a total of 600 nuclear, 600 nucleolar, and 600 somal profiles. This sample number resulted in standard errors < 10% of the mean (Bolender, '79). Planimetric measurements of nuclear, nucleolar, and somal profile areas, and nuclear profile perimeters were made with the aid of a BioQuant Image Analyzer (R&M Biometrics, Nashville, TN). From the measurements of nuclear profile areas and perimeters, a *form* factor (FF) for each nucleus was calculated using the following equation:  $FF = 4\pi \times \text{area}/\text{perimeter}^2$  (Diamond et al., '82; Buschmann and LaVelle, '83). A FF of 1 indicates a circle: a value of 1 indicates the degree of departure from a circle (A FF of 0 equals a straight line). Statistical analysis of the data was done using two-way ANOVA and the Student-Newman-Keuls test at  $P < .05$  (Sokal and Rohlf, '81).

### Distribution Analysis

From the initial, informal ultrastructural analysis,  $E_2$ -induced changes in VMN neurons were observed which included (1) increased nuclear size, (2) altered

**Figure 49.1**

Changes in ventromedial (VMN) neurons following 2 hours of  $E_2$  treatment. *A.* Control VMN neuron from sham-operated, ovariectomized (OVX) rat. The nucleus (Nu) is typically small and nonspherical with extensive invaginations of the nuclear envelope. Small clumps of heterochromatin (examples indicated by small arrows) are scattered within the nucleoplasm and along the nuclear envelope. A thin rim of cytoplasm (arrows) surrounds the nucleus. Scale bar = 1  $\mu$ m.  $\times 12,000$ . *Inset:* high-magnification view of two small clumps of heterochromatin commonly found in nucleoplasm with associated perichromatin granules (arrowheads).  $\times 50,000$ . *B.* VMN neuron after 2 hours of  $E_2$  administered to OVX rats. Transformation in the shape of the nucleus (Nu) toward spherical and pronounced nuclear and nucleolar (N) swelling have occurred. Fewer clumps of heterochromatin are scattered throughout the nucleoplasm. A single large heterochromatin clump (small arrow) is commonly observed in both the  $E_2$ -treated and control neurons. Stacked RER (arrow) cisternae within the enlarged cytoplasm (C) were frequently found in the  $E_2$ -treated neurons.  $\times 12,000$ .

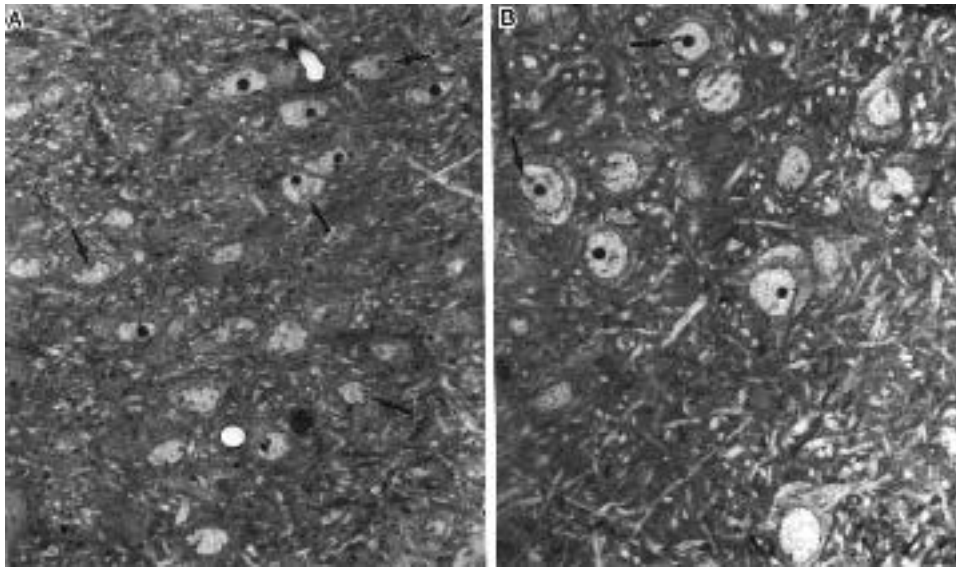
nuclear shape, (3) loss of a heterochromatin clump lining the nuclear envelope, (4) increased nucleolus-associated chromatin, (5) a more uniform, less-clumped nucleoplasm, and (6) presence of stacked RER. These six parameters were therefore selected for subsequent quantitation. Each neuron per experimental or control group (25/rat = 75/group) was examined for occurrence of each of the six parameters. The frequencies of occurrence of each of the six cellular parameters within a total population per experimental or control group of 75 neurons were then calculated. Based on these calculated frequencies and assuming mutual independence of the parameters, the rules of probability theory were applied in calculating how many neurons would be likely to contain zero, one, two, three, four, five, or six parameters. These predicted distributions of how many neurons might contain zero to six parameters were then compared to the actual distributions of neurons containing zero to six parameters. Statistical analysis of the data was done

using the Komolgorov-Smirnov test at  $P < .05$  (Siegel, '56).

## Results

### Ultrastructural Changes

After 2 hours of  $E_2$  treatment, distinct changes were observed in the nucleus. As illustrated in figure 49.1A, the nuclei in the control neurons (from sham-operated, OVX rats) tended to be ellipsoid-shaped with many small invaginations of the nuclear envelope (NEI) present. The nucleoplasm was heterogeneous in character, with numerous small clumps of heterochromatin scattered throughout the nucleoplasm and along the inner membrane of the nuclear envelope (inset, figure 49.1). In the experimental group, this 2-hour  $E_2$  treatment tended to transform nuclear shape toward spherical, with less extensive NEI present (figure 49.1B). Nuclear enlargement was also clearly evident. A decrease in the scattered small clumps of heterochromatin



**Figure 49.2**

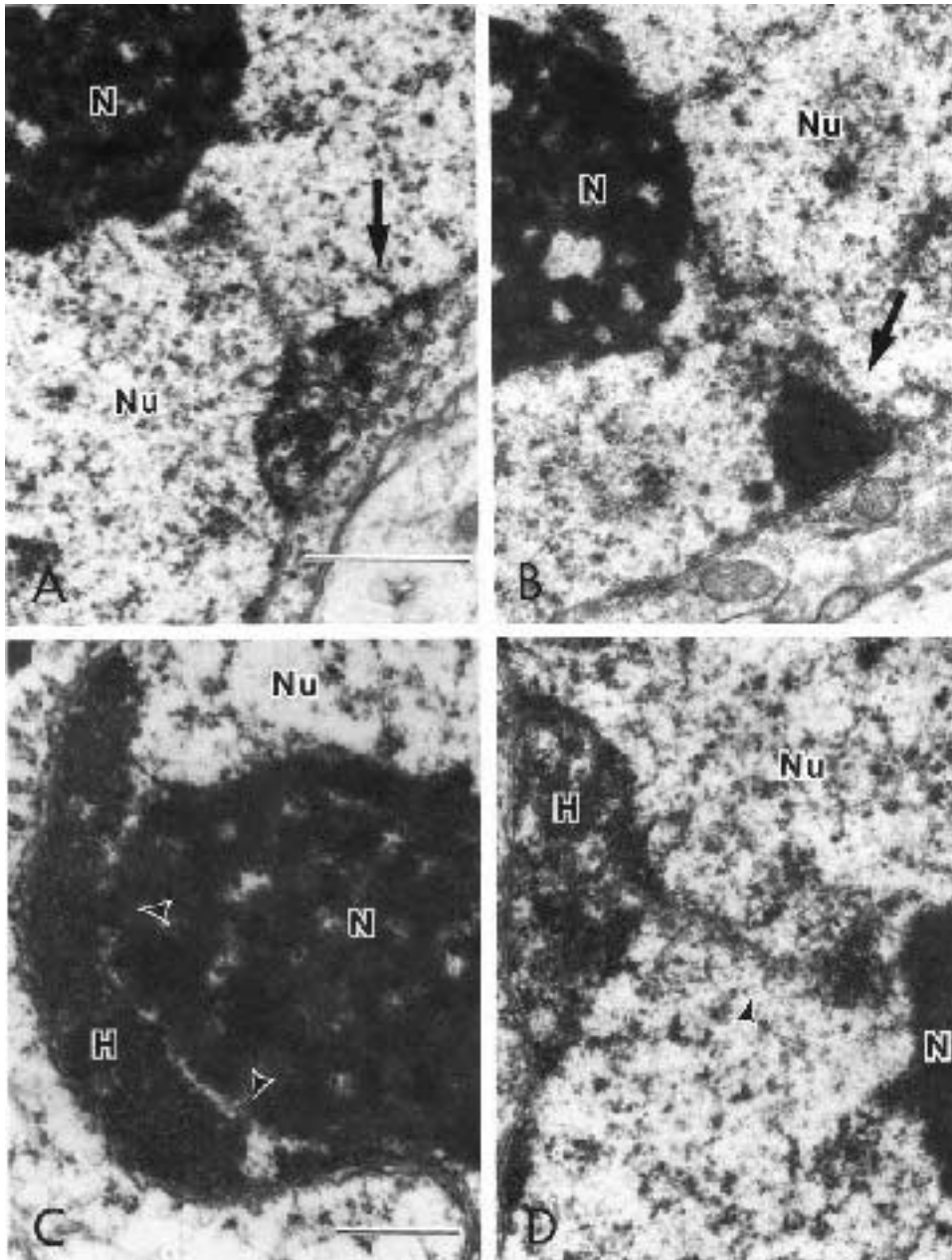
Photomicrographs of representative fields of VMN neurons following 2 hours of  $E_2$  treatment. *A.* Control VMN neurons from sham-operated, OVX rats. Nuclear profile shape is generally ellipsoid. Extensive nuclear envelope invaginations are present in many neurons (arrows).  $\times 640$ . *B.* VMN neurons after 2 hours of  $E_2$  administered to OVX rats. Nuclear profile shape has been transformed from ellipsoid toward spherical. Nuclear envelope invaginations, although still present, are less extensive than in control neurons (arrows).  $\times 640$ .

within the nucleoplasm, but not along the inner membrane of the nuclear envelope, was observed (figure 49.1B). No overt changes in ribonucleoprotein (RNP)-containing components of the cell nucleus, including interchromatin granule clusters and perichromatin granules, were noted. While the nucleolus appeared swollen, overt indications of altered nucleolar morphology were not observed. Somal swelling was also evident. Representative fields of ventromedial neurons are illustrated in the low-power photomicrographs taken from OVX control animals (figure 49.2A) and 2-hour  $E_2$ -treated animals (figure 49.2B). Small neurons with ellipsoid, invaginated nuclear profiles were present under OVX conditions (figure 49.2A), whereas 2 hours of  $E_2$  treatment resulted in enlarged cells with round, swollen, less extensively invaginated nuclear profiles (figure 49.2B).

In both control and estrogen-treated neurons, one or more large heterochromatin clumps were often found connected to the nuclear envelope (figure 49.3A) by multiple attachment sites. These nuclear envelope-attached clumps varied morphologically, being either vacuolated and spread out along the nuclear envelope (figure 49.3A), or rounded and condensed (figure 49.3B). Frequently, the nucleolus was either directly attached to the nuclear envelope via the heterochromatin (figure 49.3C) or indirectly connected via elongated strands of electron-dense fibrillar material originating from a large clump attached to the nuclear envelope (figure 49.3D).

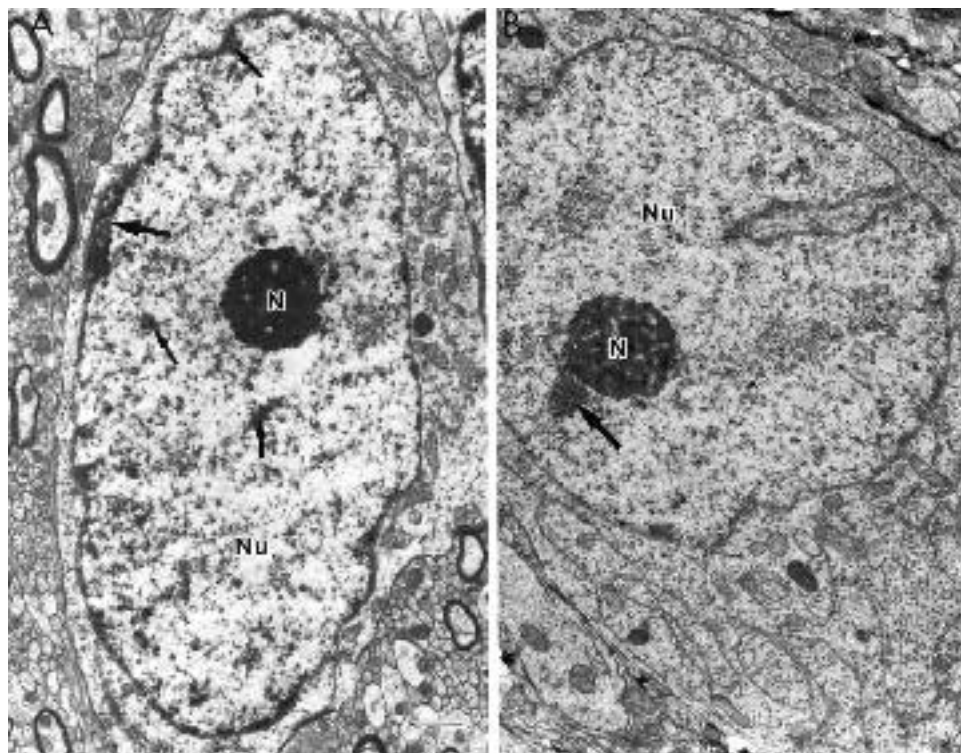
After a *discontinuous*, behaviorally sufficient (Parsons et al., '82a) paradigm of estrogen treatment, consisting of 2 hours of  $E_2$  administered via subcutaneous silastic implants followed by removal of the implants for 7 hours and reinsertion of the capsules for 2 additional hours, comparison of control (figure 49.4A) and estrogen-treated (figure 49.4B) neurons indicated a different pattern of changes from those found after 2 hours of  $E_2$ . Although altered nuclear shape, less extensive NEI, and enlarged nuclei still appeared after the discontinuous treatment (figure 49.4B), experimentally induced differences were not as sharply defined as after the single 2-hour dose of  $E_2$ . However, progressive loss of the small clumps of heterochromatin both within the nucleoplasm and along the inner membrane of the nuclear envelope had occurred (figure 49.4B). In addition, a decrease in the occurrence of the large, single heterochromatin clump along the nuclear periphery and an increase in the presence of nucleolus-associated chromatin (figure 49.4B) were apparent in the discontinuous  $E_2$ -treated neurons. Somal swelling was still apparent.

As illustrated in figure 49.5, several different forms of rough endoplasmic reticulum (RER) were found in the cytoplasm of both control and estrogen-treated neurons. Commonly, stacks of RER (a stack was defined as three or more parallel RER cisternae), closely associated with numerous polysomes (figure 49.5A), were observed more frequently in estrogen-treated neurons than control neurons. Single dilated,



**Figure 49.3**

Examples of various forms of large heterochromatin clumps attached to the nuclear envelope in ventromedial neurons. *A.* The heterochromatin clump (*arrow*) is spread out, vacuolated, and attached at multiple sites among the nuclear envelope. Nu, nucleus; N, nucleolus. Scale bar = 1  $\mu\text{m}$ .  $\times 26,400$ . *B.* Here, the heterochromatin clump (*arrow*) is rounded and condensed.  $\times 26,400$ . *C.* The nucleolus (N) is often found at the periphery of the nucleus (Nu), attached at numerous points (*arrowheads*) to heterochromatin (H) lining the inner membrane of the nuclear envelope. Scale bar = 0.5  $\mu\text{m}$ .  $\times 40,000$ . *D.* A high magnification of A illustrating the elongated strands of heterochromatin (*arrowhead*) interconnecting the heterochromatin (H) at the nuclear (Nu) periphery with the nucleolus (N).  $\times 40,000$ .



**Figure 49.4**

Changes in ventromedial (VMN) neurons following 2-hour  $E_2$ /7-hour off/2-hour  $E_2$ . *A*. Control VMN neuron from sham-operated, OVX rat. The nucleus (Nu) is nonspherical with small clumps of heterochromatin (examples indicated by *small arrows*) scattered throughout the nucleoplasm and along the nuclear envelope. A single, large heterochromatin clump (*arrow*) is often observed. The small nucleolus (N) usually lacks well-defined masses of nucleolus-associated chromatin. Scale bar = 1  $\mu$ m.  $\times 12,000$ . *B*. VMN neuron after 2-hour  $E_2$ /7-hour off/2-hour  $E_2$  treatment. Alterations in the shape of the nucleus (Nu) are not as evident as after 2 hr of  $E_2$ . Progressive loss of small heterochromatin clumps within the nucleoplasm and along the nuclear envelope has occurred. A prominent mass of nucleolus-associated chromatin (*arrow*) is present.  $\times 12,000$ .

and often multiple-branched, strands of RER budding off the outer membrane of the nuclear envelope (figure 49.5B) occurred in both experimental and control neurons.

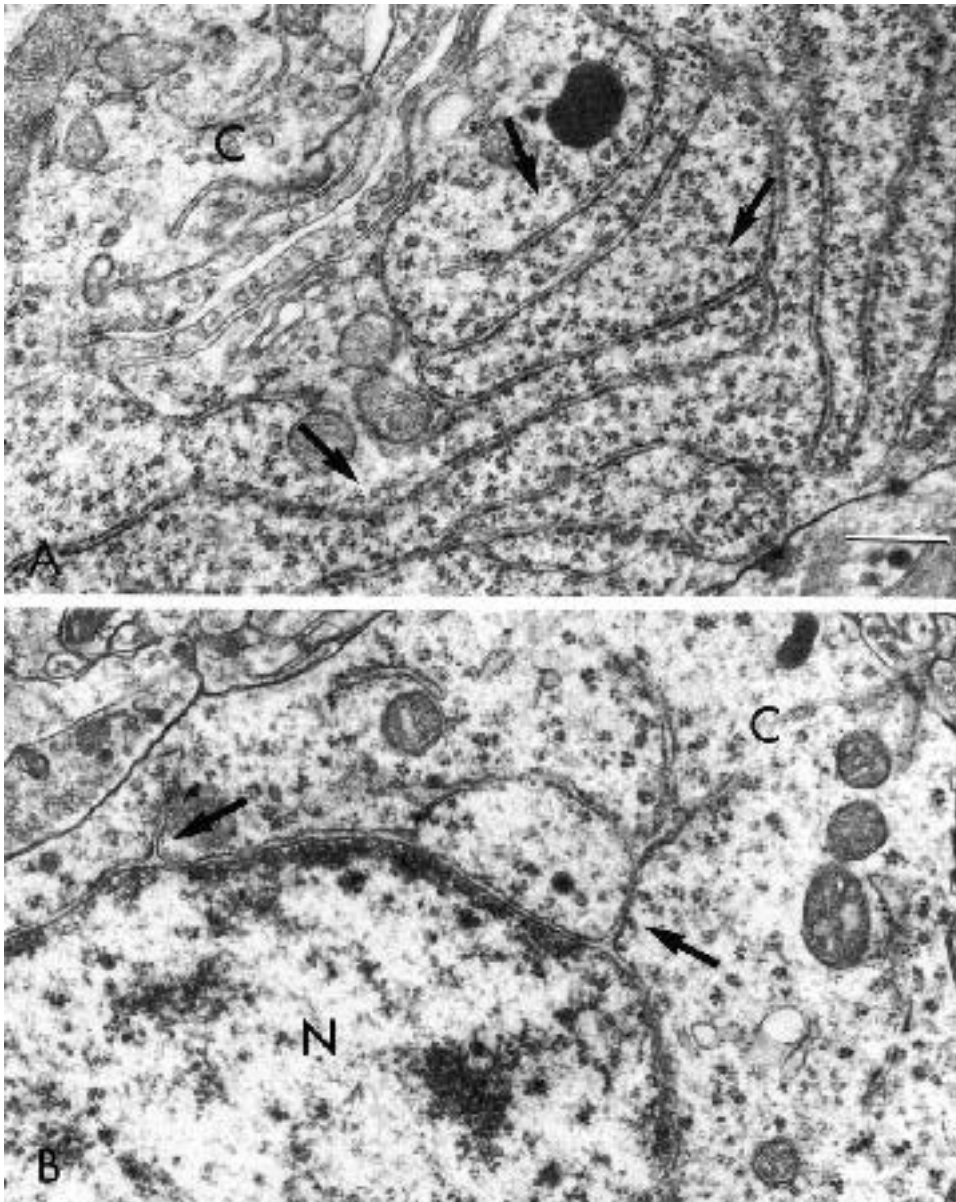
#### Morphometric Changes

From the results of the initial ultrastructural analysis, nuclear shape, nuclear perimeter (amount or length in  $\mu$ m of nuclear envelope), nucleolar size, and somal size appeared to be affected by steroid hormone treatment. To provide quantitative data regarding these changes, camera lucida tracings were made and analyzed with the BioQuant Image Analyzer as described in the Methods section. The results are summarized in table 49.1.

After 2 hours of  $E_2$ , a change in nuclear profile shape, from nonspherical to spherical, had occurred. The form factor value for the 2 hour estrogen-treated nuclear profiles had significantly increased ( $P < .05$ ) as compared to the paired control value. Accompanying this increase in form factor value were significant increases in nuclear profile area and perimeter, nucleolar profile area, and somal profile area ( $P < .05$ ).

These changes represented a 35% increase in nuclear profile area, a 10% increase in nuclear profile perimeter, a 28% increase in nucleolar profile area, and a 35% increase in somal profile area, as compared to the averaged control values.

After the discontinuous  $E_2$  treatment, no significant changes in form factor values (which being less than 1 indicated nonspherical nuclear shape) were observed between  $E_2$ -treated and paired control. Significant *increases* ( $P < .05$ ) in nuclear profile area and perimeter, and somal profile area, were found. These changes represented a 28% increase in nuclear profile area, a 7% increase in nuclear profile perimeter, and a 24% increase in somal profile area, as compared to the averaged control values. No significant differences between  $E_2$ -treated and control nucleolar profile areas were observed. Although the value for nucleolar area in the discontinuous  $E_2$ -treated group (table 49.1) was highest, the range of variation among the individual observations within this group (each observation represented the mean of 50 nucleolar profile areas from one animal) was also greatest.

**Figure 49.5**

Examples of rough endoplasmic reticulum (RER) in ventromedial neurons. *A.* Stacked RER (*arrows*) with interconnecting cisternae and closely associated with numerous polysomes are present within the cytoplasm (C) 2× more often in E<sub>2</sub>-treated neurons than control neurons. Scale bar = 0.5  $\mu$ m.  $\times 36,000$ . *B.* Budding off the outer membrane of the nuclear envelope are single and multiply branched strands of RER (*arrows*). Similar numbers of E<sub>2</sub>-treated and control neurons had nuclear envelope-RER connections.  $\times 36,000$ .

**Table 49.1**Effects of E<sub>2</sub> on nuclear shape, nuclear perimeter, nuclear area, nucleolar area, and somal area<sup>1</sup>

	2-Hour E <sub>2</sub> vs. Control		2-Hour E <sub>2</sub> /7-Hour off/2-Hour E <sub>2</sub> vs. Control	
	OVX	E <sub>2</sub>	OVX	E <sub>2</sub>
Nuclear shape (FF) <sup>2</sup>	0.72 ± 0.03	1.00* ± 0.08	0.78 ± 0.01	0.82 ± 0.04
Nuclear area (μm <sup>2</sup> )	52.93 ± 2.14	73.96* ± 4.04	56.90 ± 0.74	69.14* ± 3.79
Nuclear perimeter <sup>3</sup> (μm <sup>2</sup> )	30.74 ± 0.69	33.58* ± 1.10	30.34 ± 0.46	32.51* ± 0.17
Nucleolar area (μm <sup>2</sup> )	4.72 ± 0.24	6.06* ± 0.46	5.23 ± 0.28	6.16 ± 0.48
Somal area (μm <sup>2</sup> )	127.81 ± 7.86	185.16* ± 10.77	147.42 ± 6.80	169.87* ± 8.47

<sup>1</sup> All data were collected using a BioQuant Image Analyzer. Statistical analysis was accomplished using a two-way ANOVA and the Student-Newman-Keuls test at  $P < .05$ .

\* Indicates significant difference from the paired, sham-operated OVX controls. Standard errors are indicated below actual values.

<sup>2</sup> Form factor (FF) =  $4\pi \times \text{area}/\text{perimeter}^2$ . A FF value of 1.0 = a circle; 0.0 = a straight line.

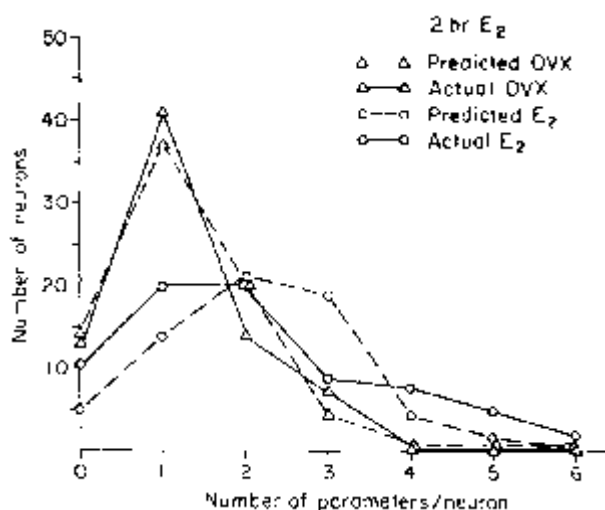
<sup>3</sup> Nuclear perimeter, the length in μm of nuclear envelope.

### Distribution Analysis

On the basis of the initial ultrastructural analysis, there appeared to be a separate subpopulation of neurons within each group that exhibited a certain unique profile. Common morphological features of this subgroup included large, rounded nuclei with less extensive invaginations and one or more of the following: nucleolus-associated chromatin, loss of a large chromatin clump at the nuclear envelope, homogeneous nucleoplasm, and stacked RER. Between 30 and 45% of the cells in the E<sub>2</sub>-treated groups and 10–13% of the cells in the OVX control groups had many of these ultrastructural features. It had also been determined, by analysis of the ultrastructural and the morphometric data, that E<sub>2</sub>-related features of VMN neurons included the following six parameters: (1) increased nuclear size, (2) altered nuclear shape, (3) loss of a heterochromatin clump lining the nuclear envelope, (4) increased nucleolus-associated chromatin, (5) a more uniform, less-clumped nucleoplasm, and (6) presence of stacked RER. In order to see if these E<sub>2</sub>-induced changes occurred without respect to each other in individual cells or if they co-occurred more frequently than expected, we undertook an analysis of the distribution of neurons containing zero to six of these parameters.

Comparisons were first made between the *actual* distributions of cells containing zero to six parameters in the 2-hour E<sub>2</sub> and the sham-implanted control groups (figure 49.6), and between the 2-hour E<sub>2</sub>/7-hours off/2-hour E<sub>2</sub> and the sham-implanted, OVX control groups (figure 49.7) using the Komolgorov-Smirnov test. In both comparisons, significant differences ( $P < .05$ ) between the distributions of the E<sub>2</sub>-treated groups and the paired control groups were found.

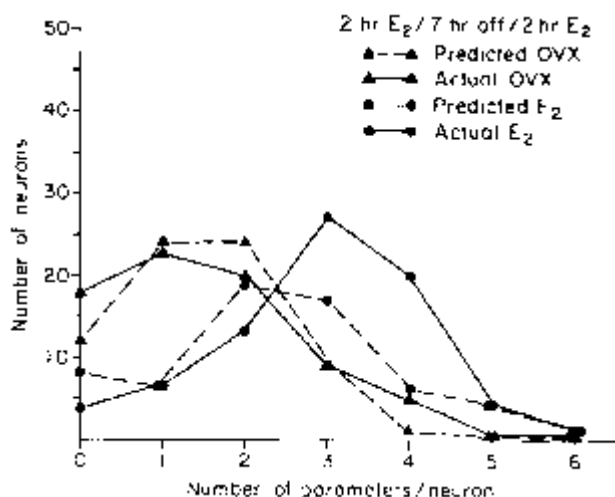
Then, the possibility of significant co-occurrences among estrogen-induced changes was explored in the following analysis. Assuming mutual *independence* of each of the six parameters (table 49.2), *theoretical* distributions of the cells containing zero to six parameters

**Figure 49.6**

Predicted and actual distributions of the number of neurons (total = 75/group) in the 2-hour E<sub>2</sub> and sham-operated, OVX control groups containing zero to six of the cellular parameters listed in table 49.2. Using the Komolgorov-Smirnov test at  $P < .05$ , significant differences were noted between the predicted and actual distributions of the E<sub>2</sub> group but not between the predicted and actual distributions of the OVX control group. Significant differences were also noted between the actual distributions of the E<sub>2</sub> and the OVX control groups.

were generated by multiplying simple probabilities of occurrence of the individual parameters, for each of the experimental and control groups.

These *predicted* distributions of cells containing zero to six parameters were compared with the *actual* distributions of cells containing zero to six parameters using the Komolgorov-Smirnov test at  $P < .05$ . No significant differences were observed between the predicted and actual distributions of cells for either the 2-hour (figure 49.6) or the 2/7/2-hour (figure 49.7) sham-operated OVX control group. However, significant differences were observed between predicted and actual distributions of cells in both E<sub>2</sub>-treated groups (2 hours, figure 49.6; 2/7/2 hours, figure 49.7).

**Figure 49.7**

Predicted and actual distributions of the number of neurons (total = 75/group) in the 2-hour E<sub>2</sub>/7-hour off/2-hour E<sub>2</sub> and sham-operated, OVX control groups containing zero to six of the cellular parameters listed in table 49.2. Using the Kolmogorov-Smirnov test at  $P < .05$ , significant differences were noted between the predicted and actual distributions of the E<sub>2</sub> group but not between the predicted and actual distributions of the OVX control group. Significant differences were also noted between the actual distributions of the E<sub>2</sub> and the OVX control groups.

**Table 49.2**

Actual frequencies<sup>1</sup> of selected cellular parameters<sup>2</sup>

	2-Hour E <sub>2</sub> vs. Control <sup>3</sup>		2-Hour E <sub>2</sub> /7-Hour off/2-Hour E <sub>2</sub> vs. Control	
	OVX	E <sub>2</sub>	OVX	E <sub>2</sub>
Spherical nuclear shape	0.12	0.31	0.21	0.64
Enlarged nucleus	0.13	0.44	0.24	0.69
Presence of NAC <sup>4</sup>	0.13	0.15	0.08	0.23
Homogeneous nucleoplasm	0.07	0.37	0.30	0.36
Presence of stacked RER <sup>4</sup>	0.05	0.17	0.07	0.12
Absence of chromatin clump at NE <sup>4</sup>	0.62	0.61	0.56	0.79

<sup>1</sup> Actual frequencies given here represent the percentage of neurons (from a total of 75 per group) displaying each individual parameter.

<sup>2</sup> See text for explanation of selection of these parameters.

<sup>3</sup> Controls are paired, sham-operated OVX animals.

<sup>4</sup> NAC, nucleolus-associated chromatin; RER, rough endoplasmic reticulum; NE, nuclear envelope.

Finally, with respect to the *actual* distributions of cells containing zero to six parameters, the control groups (actual 2-hour OVX vs. actual 2/7/2-hour OVX, see figures 49.6, 49.7) did not differ from each other, whereas, the two E<sub>2</sub>-treated groups (actual 2-hour E<sub>2</sub> vs. actual 2-hour E<sub>2</sub>/7-hour off/2-hour E<sub>2</sub>, see figures 49.6, 49.7) did significantly differ from each other ( $P < .05$ ).

## Discussion

Previous morphological studies of the effects of chronic exposure (15 days) to estrogen on ventromedial hypothalamic neurons of OVX rats have documented extensive changes in components of the protein biosynthetic apparatus of the cell that include rough endoplasmic reticulum stacking, increased presence of dense-core vesicles, and increased nucleolus-associated chromatin (Cohen and Pfaff, '81; Cohen et al., '84). Similar changes in the cytoplasmic organelles associated with protein synthesis were observed in VMN neurons of OVX rats 2 days after injection of estradiol benzoate (Carrer and Aoki, '82). Biochemical studies of steroid hormone action on target tissue have provided evidence that altered RNA and protein synthesis occur within hours after hormone administration (Means and Hamilton, '66; O'Malley and Means, '74). The results of the present investigation provide the first evidence that fine structural and conformational changes in the nuclei of rat ventromedial hypothalamic neurons occur (1) within the initial 2 hours of E<sub>2</sub> treatment, (2) after the minimal E<sub>2</sub> exposure time necessary to facilitate behavioral responses, and (3) in a coordinated interdependent manner. Cellular parameters observed in this study to be affected by estrogen treatment included the shape and size of the nucleus, character of the nucleoplasm, size of the nucleolus, presence of nucleolus-associated chromatin, loss of a large heterochromatin clump, amount of stacked RER, and somal size. Importantly, although there is no known specific cellular marker of an estrogen-concentrating neuron at the EM level, the sampling strategy and distribution analysis adopted in this investigation allowed us to establish a profile of an estrogen-responsive neuron that consists of a large, round nucleus with 1 or more of the above-listed parameters. A major finding of this investigation was that the percentage of neurons (between 30 and 45%) within the VMN of E<sub>2</sub>-treated animals that exhibited this unique profile approximated the percentage of neurons demonstrated autoradiographically to concentrate estrogen (Morrell and Pfaff, '83). These data are in accord with, and extend the findings of, the aforementioned reports documenting E<sub>2</sub>-induced structural alterations in VMN neurons.

One of the most striking changes visually noted, and morphometrically verified, after the first 2 hours of estrogen treatment was the transformation in nuclear shape toward spherical. Concomitant with this shape change was a swelling reaction in the nucleus and the nucleolus. That the nucleus is a dynamic structure, responsive to the physiological state of the cell, has been widely documented (for review, see Berezney, '79). In many eukaryotic cell types, increases in nuclear and nucleolar size have been found to be associated with corresponding increases in the level of extranucleolar and nucleolar transcriptional activity (Merriam, '69; Busch and Smetana, '70). Qualitative, but not quantitative, evidence of steroid hormone-induced transformation of nuclear shape, from nonspherical, invaginated to spherical, smooth nuclear profiles, exists in cat oviduct epithelial cells (Bareither and Verhage, '81). While the exact nuclear acceptor site for binding of the activated hormone-receptor complex is currently a subject of controversy (Spelsberg et al., '83), strong evidence exists which suggests that the *in situ* nuclear matrix is involved in the activational effects of steroid hormones on target tissues (Barrack and Coffey, '82; Ciejek et al., '83; Simmen et al., '84). A relationship between conformational changes in the *in situ* matrix and conformational changes in the nucleus has been postulated (Berezney, '84). It is plausible, therefore, that the spherical, enlarged nuclei observed in the present study after estrogen treatment for 2 hours morphologically reflect expansion of the nuclear matrix as it undergoes structural reorganization necessary for gene expression. Alternatively, it is possible that changes in the osmotic conditions of the cell as a result of hormone treatment could account for the nuclear reaction. In this regard, Wunderlich et al. ('74) have shown that the isolated nuclear matrix is capable of alternate expansion/contraction when the concentration of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  in the medium is varied. Thus, a relationship between nuclear matrix expansion and osmotic fluctuations may exist, supporting a role for the nuclear matrix in maintenance of nuclear shape and size. In this regard, Nicolini et al. ('84) recently examined phase transitions in nuclei and chromatin in order to determine what controls nuclear size. Their results suggest that nuclear volume and chromatin structure are directly affected by pH, electric charge, and ionic quantity. Furthermore, these investigators suggest that the phase transitions undergone by chromatin may in turn determine organization and function of the nuclear matrix and, subsequently, nuclear size and shape.

The early and widespread  $\text{E}_2$ -induced decondensation of heterochromatin clumps scattered throughout the nucleoplasm that was observed in this study implies an increase in elements of the interchromatinic spaces, resulting in a homogeneous-appearing or "filled"-in

nucleoplasm. It is now well established that the interchromatin domain of the nucleus contains actively transcribing DNA closely associated with nascent RNA in the form of ribonucleoprotein (RNP) fibrils and granules (Puvion-Dutilleul, '83). Thus, the ultrastructural evidence in this study suggests that an early effect of  $\text{E}_2$  on VMN neurons may be stimulation of non-nucleolar RNA synthesis. Similar gene-activational effects of steroid hormone on recognized brain target areas have been demonstrated by Kelner et al. ('80), who found increases in RNA polymerase II activity in hypothalamic cell nuclei following estradiol administration, and Dokas and Liauw ('84), who found that corticosterone applied to hippocampal slices resulted in an early increase in tritiated uridine incorporation into heterogeneous nuclear RNA. Chromatin dispersion in rat endometrial cells was ultrastructurally noted and correlated biochemically to an increase in transcription by Vic et al. ('80). Further, these investigators were able to block  $\text{E}_2$ -induced nucleoplasmic chromatin decondensation by administration of cordycepin, which interferes with mRNA processing by blocking polyadenylation.

As mentioned, the presence of nucleolar swelling has been found to be correlated with increased rRNA synthesis. Usually, however, significant alterations in nucleolar substructure involving the fibrillar, granular, and vacuolar regions (Jones, '83) accompany the enlargement in size. Overt changes within the nucleolus in response to estradiol were *not* observed in this study. The possibility that subtle, distributive changes in components of the nucleolus may be present cannot be ruled out without more extensive stereological analysis of the individual intranucleolar regions. It remains questionable, therefore, as to whether or not the nucleolar swelling reaction signifies altered rRNA synthesis. Since a nucleolar protein framework has been shown to exist (Franke et al., '81) which connects to the nuclear envelope via the internal nuclear matrix (Bernhard, '69), it may be that nucleolar skeletal changes occur in concert with nuclear matrix changes and are responsible for the increased size of the nucleolus.

After the discontinuous treatment with  $\text{E}_2$ , which involved a second 2-hour pulse of  $\text{E}_2$  7 hours after the first 2-hour hormone treatment, no significant quantitative changes in nuclear shape were observed although nuclear swelling was still present. This indicates that nuclear shape changes receded between the two pulses of  $\text{E}_2$  and were not reactivated by the second 2-hour  $\text{E}_2$  treatment. Why the *second* 2-hour exposure to  $\text{E}_2$ , which is critical for elicitation of the subsequent behavioral response (Parsons et al., '82a,b), did not cause any transformation in nuclear shape is not known but is important in view of evidence suggesting that there may be separate initial and later components of  $\text{E}_2$

action (Parsons et al., '81). In this regard, the nuclear swelling present after the second exposure to  $E_2$  may have been a consequence of the first 2 hours of  $E_2$  that is still manifested 9 hours (7 hours off and 2 hours  $E_2$ ) later. Although statistically not significantly different from the 2-hour  $E_2$  group, the trend in all parameters quantified after the second 2 hours of  $E_2$  was toward a return to control values. In order to answer this question, it will be necessary in future experiments to examine the nuclei of VMN neurons 9 hours after only one 2-hour exposure to  $E_2$ . Nonetheless, the differential effects of the early and later 2-hour pulses of  $E_2$  on nuclear shape suggest that separate and distinct events may be occurring with the two treatments of  $E_2$ .

In addition to the continued decondensation of scattered small clumps along the nuclear envelope as well as within the nucleoplasm, a correlative decrease in a large heterochromatin clump along the nuclear envelope and increase in nucleolus-associated chromatin was observed after the discontinuous treatment with  $E_2$ . This correlation and the ultrastructural evidence presented here for interconnections between the two structures are noteworthy in view of the observation that nucleolar protuberances composed of DNA (Chung et al., '84) occur twice as often in VMN neurons chronically treated with  $E_2$  as in OVX controls (Cohen et al., '84). Intriguing relationships were also noted between the outer membrane of the nuclear envelope and the rough endoplasmic reticulum. After both  $E_2$  treatment paradigms, stacked RER was more frequently observed in the  $E_2$ -treated neurons than in the controls. In addition, the morphometric evidence indicated that  $E_2$  treatment results in increased production of nuclear envelope. These estrogen-induced increases in the amount of nuclear envelope and the presence of stacked RER, along with evidence for extensive structural continuity between them, suggest changes in the production and/or redistribution of these two membrane systems that may reflect alterations in the amount and type of proteins synthesized. Similar observations have been documented in developing (LaVelle and Buschmann, '83) and axotomized hamster facial motor neurons (Jones and LaVelle, '85). These investigators correlated changes in the amount of nuclear envelope to fluctuating levels of Nissl substance (multiple stacks of RER in these neurons) produced by both neuronal maturation and axotomy.

On the basis of the ultrastructural and morphometric analyses, we observed a number of neurons within each  $E_2$ -treated or control ventromedial nucleus that could be readily distinguished by several morphological features. These included large, rounded nuclei with less extensive invaginations and one or more of the following: nucleolus-associated chromatin, homogenous

nucleoplasm, loss of a large heterochromatin clump lining the nuclear envelope, and stacked RER. Between 30 and 40% of the cells in the  $E_2$ -treated groups and between 10 and 13% of the cells in the OVX, sham-operated control groups displayed this profile. Morrell and Pfaff ('83) have demonstrated, by steroid hormone autoradiographic techniques, that approximately 30% of the neurons in the VMN concentrate estrogen. Van Houten and Brawer ('78a,b), in extensive analyses of the morphology of the VMN in the adult male rat hypothalamus, discovered regional variations in the ultrastructure of individual neurons. Since the percentage of morphologically distinct neurons within the VMN of  $E_2$ -treated animals in the present investigation was quite consistent with the findings of Morrell and Pfaff ('83), we further investigated the relationship of six  $E_2$ -related structural parameters (see table 49.2) to steroid hormone action on VMN neurons by analyzing the distribution of neurons within each experimental or control group containing zero to six of the parameters. The results indicated that significant differences in the number of VMN neurons containing from zero to six of the parameters existed between the  $E_2$ -treated and OVX conditions, with there being a shift toward increased numbers of individual parameters per neuron following the administration of estrogen. Furthermore, the differences between predicted and actual distributions of neurons containing zero to six parameters under hormone but *not* OVX conditions demonstrate that (1) in the hormone-withdrawn state, the parameters occurred independently of each other or randomly whereas (2) in the hormone-activated state, the parameters co-occurred more frequently than predicted, meaning that estrogen induced structural interrelationships between the cellular parameters selected for study.

By examining the frequencies of occurrence of each parameter in the morphologically distinct subpopulation of neurons within the  $E_2$ -treated groups, a cascade of events can be inferred, involving first nuclear swelling followed by alteration in nuclear shape and decondensation of heterochromatin in the nucleoplasm. These occur after 2 hours of hormone administration. After the second pulse of  $E_2$ , we see the correlated increase in nucleolus-associated chromatin and the decrease in the large heterochromatin clump lining the nuclear envelope. What this series of events implies chemically in terms of protein synthesis following steroid hormone action on VMN neurons is under investigation.

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Previous studies have demonstrated that intrahypothalamic injection of the serotonin (5-HT) neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) results in the facilitation of lordosis behavior in ovariectomized (OVX), estrogen-treated rats [1]. The facilitation of lordosis behavior can be correlated to the degeneration of 5-HT axons in the ventromedial hypothalamic nucleus (VMN) [2, 3], an area which has been shown to be critical for the display of gonadal-steroid-dependent female sexual behavior [4, 5] and which contains a high concentration of estrogen-binding sites [6]. The mechanisms which underlie 5,7-DHT-induced facilitation of lordosis probably involve an interaction between 5-HT and estrogen in the VMN, since 5,7-DHT appears to lower the threshold of estrogen needed to elicit lordosis. However, it appears that 5,7-DHT injection does not alter the number of estrogen receptors in the VMN [7].

More recently we have investigated the effects of gonadal steroid manipulation on dendritic spine density on neurons in the VMN [8]. Using Golgi impregnation it was demonstrated that dendritic spine density in the VMN fluctuated during the estrous cycle and after estrogen administration to OVX rats. Interestingly, dendritic spine density was greatest when lordosis behavior was manifested, for example during proestrus in the intact animal and after estrogen administration to OVX rats.

Given our hypothesis that there is an interaction between 5-HT and estrogen in 5,7-DHT-induced lordosis, the purpose of the present study was to examine the interaction between 5,7-DHT and estrogen on dendritic spine density in the VMN. The effect of 5,7-DHT on spine density in the VMN was determined in intact rats of both sexes to assess possible sex differences. The interaction between estrogen and 5,7-DHT treatment was determined in gonadectomized (GDX) female and male rats. Male rats were examined because it has been previously demonstrated that intrahypothalamic injection of 5,7-DHT also facilitates lordosis behavior in males [9].

## Methods

Rats (225–250 g) were housed under controlled light (14 h light, 10 h dark; lights on at 5 A.M.) conditions with food and water available ad libitum.

### Experiment 1: 5,7-DHT in Intact Rats

Intact female and male rats received injections of 5,7-DHT or vehicle into the dorsolateral hypothalamus. Seven days later the rats were perfused and processed for Golgi impregnation.

### Experiment 2: Castration and Estrogen in Males

Male rats were castrated under Metofane anesthesia. Five days later the castrated animals received injections of 10 µg of estradiol benzoate (EB, in sesame oil) or oil subcutaneously for 2 consecutive days. An intact group of males was also used. All rats were perfused and processed for Golgi impregnation 48 h after the last EB injection.

### Experiment 3: Interaction between 5,7-DHT and Estrogen

Male and female rats were anesthetized once as described below, GDX and immediately thereafter given 5,7-DHT injections. Five days later the animals received either 10 µg EB or oil s.c. for 2 consecutive days, and 48 h after EB injection the animals were perfused and processed for Golgi impregnation.

### 5,7-DHT Injections

Stereotaxic injections of 5,7-DHT were made into the dorsolateral hypothalamus as described previously [2]. Briefly, animals were injected intraperitoneally with 10 mg/kg desmethylinipramine (desipramine HCl, Sigma) 30 min prior to 5,7-DHT injection. The rats were anesthetized with a mixture of ketamine (Ketalar HCl, 85 mg/kg) and Promace (1.5 mg/kg) given intraperitoneally, and 5 µg of 5,7-DHT was injected in a volume of 400 nl (in 0.9 saline and 0.02% ascorbic acid) over 8 min. The coordinates used were: (nose bar –3.3) 4.5 mm anterior, 0.6 lateral and 8.5 mm ventral to λ.

### Single-Section Golgi Impregnation

A modification of the Golgi technique of Gabbott and Somogy [10] was used, because it is rapid, reliable and the sections can be monitored during impregnation. Rats were anesthetized with ketamine and Promace (as above) and transcardially perfused with 4% paraformaldehyde and 15% picric acid in a 0.1 M phosphate buffer (pH 7.4). Following perfusion, brains were postfixed in the same fixative overnight at 4 °C. Vibratome sections (100 µm) were cut into a bath of 3% potassium dichromate (in distilled water) and subsequently incubated in the same solution (at room temperature) for 24 h. Following this the sections were briefly washed in distilled water and mounted onto uncoated glass slides. Coverslips were glued over the tissue sections and the slide assemblies incubated in 1.5% silver nitrate in distilled water in the dark for 24 h. The coverslips were then removed, sections dehydrated, cleared, mounted onto subbed slides and cover-slipped under Permount.

### Data Analysis

To analyze Golgi-impregnated neurons, camera lucida drawings were made at  $\times 1,250$  without reference to the treatment group. All neurons readily distinguishable in the VMN were used (4–6 neurons per brain; 5–10 brains per group). For spine density measurements, the primary dendrite with the greatest number of spines was traced, spines were counted and the length of the dendrite was measured with the aid of an image analysis system (Southern Micro Systems, Inc.). The entire visible length of the primary dendrite was used. Spine density was then expressed per millimeter primary dendrite. Data were analyzed by Student's *t* test or one-way analysis of variance followed by Tukey's HSD test, where appropriate.

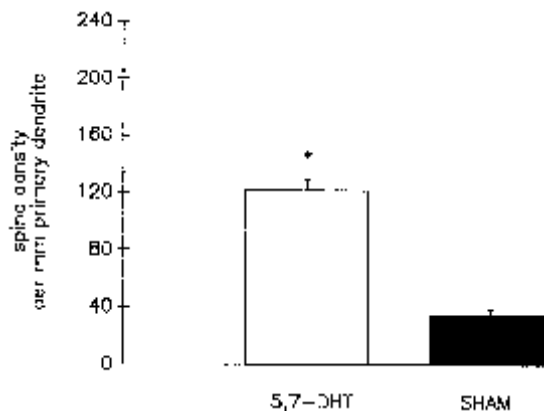
## Results

### Experiment 1: 5,7-DHT in Intact Rats

Intrahypothalamic injection of 5,7-DHT resulted in a 3- to 4-fold increase in dendritic spine density on VMN neurons of both intact female (figure 50.1) and male (figure 50.2) rats as compared to sham-operated rats.

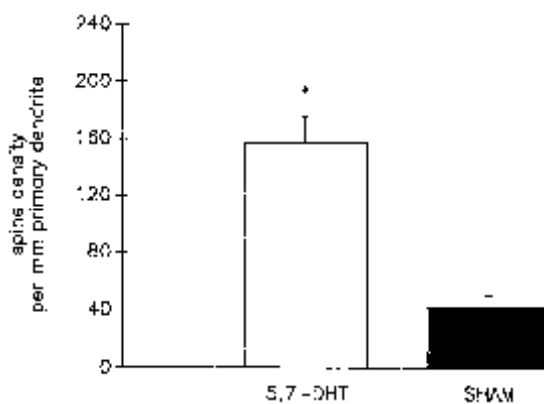
### Experiment 2: Castration and Estrogen in Males

Spine density on neurons in the VMN was significantly higher in castrated males than in intact males, and administration of 10 µg of EB partially reversed this effect (figure 50.3). There was no difference observed between 10 and 20 µg EB (data not shown), and therefore in the rest of these experiments 10 µg EB was used.



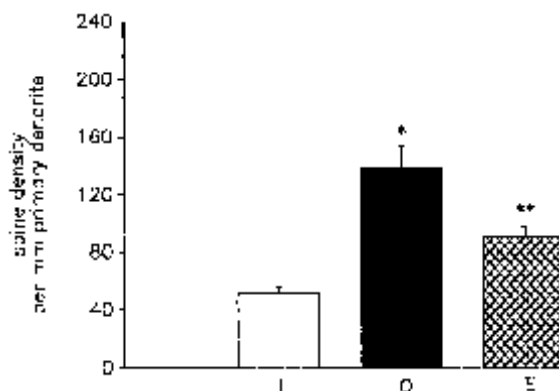
**Figure 50.1**

Bar graph illustrating the increase in dendritic spine density in VMN neurons in intact females 7 days after 5,7-DHT injection.  $n = 6-7$  brains. \* $p < 0.05$ .



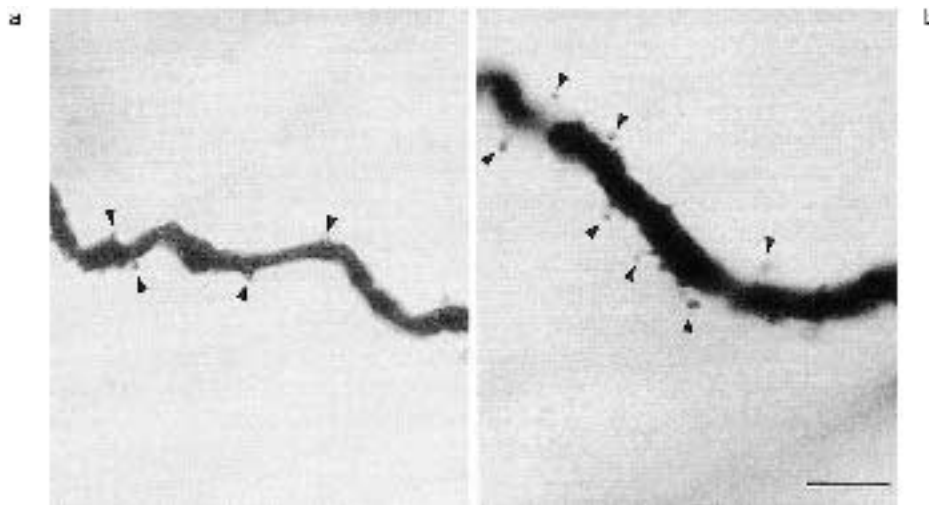
**Figure 50.2**

Bar graph illustrating the increase in dendritic spine density in VMN neurons in intact males 7 days after 5,7-DHT injection.  $n = 5-7$  brains. \* $p < 0.05$ .

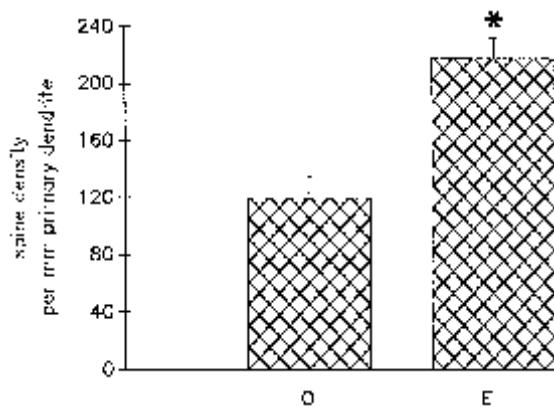


**Figure 50.3**

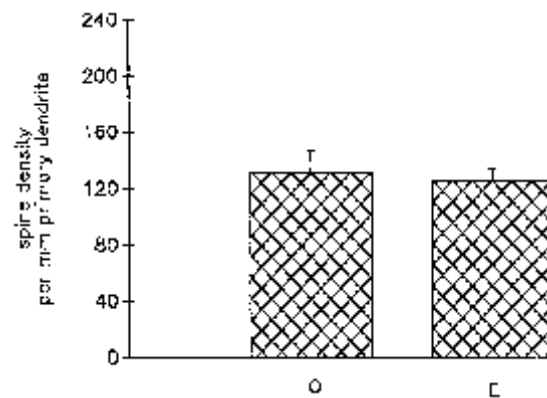
Bar graph illustrating the change in dendritic spine density in VMN neurons in the male. Data were analyzed by one-way analysis of variance followed by Tukey's HSD test. I = Intact; O = castration and oil; E = castration and estrogen; \* = significantly higher than I; \*\* = significantly different from O and E.  $n = 4-5$  brains.  $p < 0.05$ .

**Figure 50.4**

Photomicrograph illustrating dendritic spine density changes on VMN neurons after 5,7-DHT and oil (a) or estrogen (b). Arrow heads denote dendritic spines. Scale bar = 5  $\mu$ m.

**Figure 50.5**

Bar graph illustrating the increase in dendritic spine density in VMN neurons in 5,7-DHT-treated females given estrogen (E) as compared to oil (O).  $n = 6-7$  brains. \* $p < 0.05$ .

**Figure 50.6**

Bar graph illustrating the dendritic spine density in VMN neurons in the male after 5,7-DHT and oil (O) or estrogen (E).  $n = 9-10$  brains.

### Experiment 3: Interaction between 5,7-DHT and Estrogen

Injection of estrogen to OVX 5,7-DHT-treated female rats resulted in a significant increase in the density of dendritic spines on VMN neurons as compared to oil injection (figures 50.4, 50.5). This represents an increase over 5,7-DHT alone and is in the same direction as estrogen treatment. In castrated 5,7-DHT-treated males, estrogen administration did not alter spine density in the VMN (figure 50.6), in spite of the fact that estrogen treatment of GDX males decreased spine density.

### Discussion

In the present study we have demonstrated that intrahypothalamic 5,7-DHT and estrogen alter dendritic

spine density in the VMN and that there are sex differences in these effects. In intact rats we observed an increase in dendritic spine density after 5,7-DHT injection that was of similar magnitude in females and males. It has been established that intrahypothalamic 5,7-DHT injection results in de- and regeneration of 5-HT axons in the VMN [2, 3]. In addition, denervation causes an increase in 5-HT binding in the VMN that is reversed with reinnervation [11]. The increase in 5-HT binding may be due to an increase in dendritic spines which express a greater number of 5-HT receptors. Given that the number [12] and morphology [13, 14] of dendritic spines has been shown to change following deafferentation, it is possible that dendritic spines may be involved in the regeneration of 5-HT axons.

In the present context, however, the increase in dendritic spine density observed after 5,7-DHT is of

importance because, again, there is a correlation between high spine density on VMN neurons and the manifestation of lordosis. In our previous study we demonstrated that dendritic spine density on VMN neurons was greatest during proestrus in the intact rat and after estrogen administration to OVX rats [8]. The fact that 5,7-DHT injection results in a spine density similar to that observed in proestrus and after estrogen administration to OVX rats suggests that alterations in dendritic morphology play a role in mediating the lordotic response to gonadal hormones.

In male rats, gonadectomy resulted in an increase in dendritic spine density on VMN neurons as compared to intact rats. Subsequent estrogen administration decreased dendritic spine density, partially reversing the effect of gonadectomy. These results are opposite those observed in the female VMN where gonadectomy decreased dendritic spine density and estrogen administration increased spine density [8]. Sex differences in the response to gonadectomy and estrogen are to be expected, since sexual dimorphisms in the VMN have been demonstrated [15, 16]. In a recent study Segarra and McEwen [17] found that estrogen increased dendritic spine density in the VMN of juvenile and peripubertal rats. Although spine density was increased in both sexes, the increase was far greater in females. The apparent discrepancy between the latter study and our study, in which dendritic spine density in male VMN was decreased after estrogen treatment, may be due to the different ages of the rats. In other brain areas castration has been shown to alter dendritic spine density during development in the hippocampus [18], and sex differences in the number of spines on cortical pyramidal cells have been observed [19]. However, there are few data on the effects of castration in the adult male.

Given that 5,7-DHT and estrogen altered dendritic spine density in the VMN of both sexes, the next aim was to determine whether there was an interaction between 5,7-DHT and estrogen on dendritic spine density in the VMN of GDX rats. In OVX females, estrogen administration to 5,7-DHT-treated rats resulted in even greater dendritic spine density than that seen in 5,7-DHT-treated rats. These results support our hypothesis that 5,7-DHT lowers the threshold of estrogen needed to facilitate lordosis and suggests that the mechanism by which 5,7-DHT does this involves dendritic spine induction.

Results in the male rat are more difficult to interpret since males exhibit moderate lordosis responding after relatively high doses of gonadal steroids [20]. To date only the intraphypothalamic injection of 5,7-DHT has been shown to result in high lordosis quotients after relatively low levels of gonadal steroids [7]. Therefore

we were interested in determining the interaction between estrogen and 5,7-DHT on dendritic spine density in the male VMN. The increase in spine density observed in intact males after 5,7-DHT was similar to intact females. However, unlike the GDX females, in which estrogen and 5,7-DHT had an additive effect on spine density, there was no effect of estrogen treatment upon spine density in GDX 5,7-DHT-treated male rats. This suggests that the mechanism of 5,7-DHT-induced lordosis in the male differs from that in the female. There are several possible reasons for this. First, there are inherent sex differences in synaptic organization of the VMN [16]. Second, it has been demonstrated that in the VMN the estrogen-binding capacity of the female is greater than in the male [21]. Finally, there may be sex differences in the 5-HT innervation of the VMN. The latter possibility is supported by a recent study in which sex differences in monoamine oxidase activity have been found in the VMN [22]. In the VMN, monoamine oxidases, the major enzyme involved in 5-HT degradation, increases in females and decreases in males after estrogen treatment.

In conclusion, we have expanded on an earlier report in which we demonstrated a correlation between dendritic spine density on VMN neurons and lordosis in the female rat. The fact that combining estrogen and 5,7-DHT results in an additive dendritic spine density in the female suggests that these treatments affect the same neuronal population. However, whether 5-HT axons terminate directly on estrogen-concentrating cells in the VMN, or 5-HT axons and estrogen-concentrating cells project to a common third cell, cannot be determined at this point. Whatever the relationship between 5-HT and estrogen cells is, it appears that, in the female, the facilitation of lordosis may involve morphological change in the VMN.

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Plasticity in the adult nervous system has historically been thought to result from changes in the physiology and/or biochemistry of neuronal circuitry, the physical structure of which has been established during early development (for review, see Arnold and Breedlove, 1985; Gould et al., 1991). Recent evidence, however, has indicated that adult neuronal circuits are much more structurally plastic than previously thought. Several laboratories have observed naturally occurring morphologic changes in the dendrites of adult neurons that suggest ongoing modification in patterns of synaptic communication between these cells and their afferents (Meyer et al., 1978; Brandon and Coss, 1982; Burgess and Coss, 1983; Purves et al., 1986; Forger and Breedlove, 1987; Woolley et al., 1990). In this report, we present direct evidence for such synaptic plasticity in that we demonstrate naturally occurring, steroid-mediated fluctuation in the density of synapses on hippocampal pyramidal neurons in the adult mammalian brain.

Our laboratory has recently shown that, in the adult female rat, the density of apical dendritic spines on CA1 hippocampal pyramidal neurons is positively correlated with circulating levels of estradiol and progesterone. We first observed changes in dendritic spine density with experimental manipulation of these hormones (Gould et al., 1990), and subsequently at different stages of the 5 d estrous cycle when estradiol and progesterone levels are naturally high or low (Woolley et al., 1990). As dendritic spines are the sites of the majority of excitatory input to hippocampal pyramidal neurons (Westrum and Blackstad, 1962; Harris and Stevens, 1989) the implication of these findings is that the density of synaptic contacts between hippocampal pyramidal neurons and their excitatory afferents is also under the control of estradiol and/or progesterone. Further, the short-term variations in circulating levels of these ovarian steroids that occur during the estrous cycle may result in ongoing, cyclic fluctuation in synaptic connectivity between pyramidal cells and their afferents.

In order to investigate these possibilities, we have made statistical estimates of synapse density in the stratum radiatum of the hippocampal CA1 region in adult

female rats both with experimental manipulation of estradiol levels and as estradiol levels drop naturally in the shift from the proestrus to the estrus phase of the estrous cycle. In two experiments, synapse density in ovariectomized rats treated with either estradiol or oil vehicle was evaluated using, in the first case, a “conventional” estimation procedure based upon large numbers of randomly taken, area-weighted micrographs (Beaulieu and Colonnier, 1985) or, in the second case, the Disector method (Sterio, 1984; DeGroot and Bierman, 1986). The “conventional” estimation procedures are generally considered to be inferior to the Disector method (DeGroot and Bierman, 1986; but see Calverley et al., 1988) because the “conventional” techniques rely on the assumption that synaptic junctions are circular, disk-shaped structures, which they almost certainly are not (Harris and Stevens, 1989). The Disector method, on the other hand, is unbiased by the size, shape, or orientation of the test objects, in this case synapses, under study. Because we found the same relationship between treatment groups using either method and because of the general superiority of the Disector method, only the Disector was used for the estrous cycle experiment. In this third case, we compared synapse density in rats in the proestrus phase of their estrous cycle, in which estradiol levels are highest, to synapse density in rats in the estrus phase of the cycle, in which estradiol levels are lowest (Smith et al., 1975). In the experiments in which the Disector method was used, the left half of the brain was processed for electron microscopy while the right half was processed for light level analysis of dendritic spine density on CA1 pyramidal cells.

## Materials and Methods

### Animal Treatments

A total of 22 adult female Sprague–Dawley rats (275 gm) were used in this study. These rats were group housed with unlimited access to food and water.

For the experiments with experimental manipulation of estradiol levels, rats were ovariectomized under Metofane anesthesia. Three days following surgery, half of the rats received two injections of 10  $\mu$ g of

estradiol benzoate in 100  $\mu$ l of sesame oil 24 hr apart. The other half of the rats received injections of oil vehicle at these times. Forty-eight hours after the second injection, the rats were deeply anesthetized with Nembutal and transcardially perfused with 2% paraformaldehyde, 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The heads of these animals were postfixed overnight at 4°C in the perfusate solution. Brains were then removed from the cranial cavities and blocked to contain the rostral hippocampus.

For the estrous cycle experiment, daily vaginal smears were taken for over 2 weeks and only those animals with regular 5 d cycles were used. In the late afternoon (1–2 hr before “lights off”) of either the proestrus or the estrus phase of the estrous cycle, these rats were deeply anesthetized with Nembutal and transcardially perfused as described above.

### Histology

For electron microscopy, 250- $\mu$ m-thick coronal sections were cut through the rostral hippocampus using a Vibratome. Sections corresponding to Paxinos and Watson (1986) plates 31–33 were fixed in osmium tetroxide *en bloc* and stained with uranyl acetate, dehydrated in graded ethanols, and flat embedded between plastic coverslips in EMBED-812. Blocks were trimmed to contain only the apex of the CA1 pyramidal cell layer, from the stratum oriens to the stratum lacunosum-moleculare. Semithin sections were cut, stained for Nissl with toluidine blue, and drawn at 250 $\times$  using a camera lucida drawing tube. These drawings were used for orientation on ultrathin sections during data collection. Ultrathin sections were stained with uranyl acetate and lead citrate.

For experiments using the Disector method, the left half of the brain was processed for electron microscopy and the right half was processed according to a modified version of the single-section Golgi impregnation technique. Briefly, 100- $\mu$ m-thick coronal sections through the rostral hippocampus were cut into a bath of 3% potassium dichromate in distilled water using a Vibratome. These sections were then incubated overnight at room temperature in 3% potassium dichromate, rinsed in distilled water, and mounted onto ungelatinized slides. A coverslip was then glued over the sections at the four corners of the slide. These slide assemblies were incubated in 1.5% silver nitrate in distilled water overnight in the dark. Following this, sections were rinsed in distilled water, dehydrated in graded ethanols, cleared in AmeriClear, and coverslipped.

### Data Analysis

All grids containing ultrathin sections were coded prior to quantitative analysis, and the code was not broken until the analysis was complete.

**“Conventional” Estimation Procedure** Estimation of synapse density using a “conventional” method (Beaulieu and Colonnier, 1985) was performed as follows. (1) From each of two blocks of tissue for each brain, 20 electron micrographs were taken randomly from a region in the stratum radiatum between 300 and 500  $\mu$ m from the pyramidal cell layer; these micrographs covered an area of approximately 3500  $\mu$ m<sup>2</sup> per brain. (2) The number of synapses ( $N$ ), defined as having both a postsynaptic density and at least two vesicles in the presynaptic terminal no more than 0.2  $\mu$ m from the synaptic cleft, was counted. (Note that these requirements leave some synapses undetected depending upon their orientation to the cutting plane. Thus, the values reported using this method likely underestimate the actual density of synapses in this region.) Without viewing serial sections, perforated synaptic junctions transected by the cutting plane usually appear as more than one synapse. In our analysis, when a single presynaptic terminal was associated with more than one postsynaptic density on the same postsynaptic element, it was counted as a single synapse. If, however, a presynaptic terminal clearly formed synapses with more than one postsynaptic element, then this was counted as more than one synapse. Additionally, synapses on dendritic spines were counted separately from those on dendritic shafts. Since relatively few cross sections contained a spine neck or spine apparatus, spines were defined as postsynaptic processes smaller than 2  $\mu$ m<sup>2</sup>, lacking mitochondria. (3) The exact area of the sampled neuropil was measured using the Southern Micro Instruments (SMI) image analysis morphometry program. In order to normalize the sampled neuropil area that could contain synapses by excluding areas covered by large synapse-free structures, the areas of any cell bodies, myelinated processes, and dendritic processes greater than 2  $\mu$ m<sup>2</sup> were measured using the SMI morphometry program and the sum was subtracted from the total area sampled to give a corrected value for the area sampled (corr  $A$ ). Subtraction of the areas of dendritic processes greater than 2  $\mu$ m<sup>2</sup> has been determined by Harris et al. (1989) to achieve homogeneity of variance of cross-sectional areas of dendritic processes in the neuropil of stratum radiatum in the hippocampal CA1 region. (4) The length of each synapse transect was measured with the SMI morphometry system, and a mean was calculated ( $\bar{d}$ ). (5) Estimated synapse density (est  $N_v$ ) was then calculated:

$$\text{est } N_v = N / (\text{corr } A \times \bar{d}).$$

**Disector Procedure** Synapse density was estimated by the Disector method as follows (see DeGroot and Beirman, 1986, for details). (1) From each brain, six overlapping electron micrographs of three separate regions

(18 micrographs per section) within the stratum radiatum between 300 and 500  $\mu\text{m}$  from the pyramidal cell layer were taken to create three “reference” planes. Regions containing cell bodies or blood vessels were intentionally avoided. The exact areas of the regions examined ( $A_i$ ,  $i = 1, 2, 3$ ) approximately 400  $\mu\text{m}^2$ , were measured with the SMI morphometry program. (2) Micrographs of exactly the same regions were taken on an adjacent section to create three “look up” planes each necessarily of area  $A_i$ . (3) The number of synaptic transects contained in a “reference” plane but absent in the corresponding “look up” plane ( $Q_i^-$ ) was counted in order to determine the number of synapses present within the volume defined by the “reference” plane, the “look up” plane, and the distance between them ( $h_i$ ; see below). As described for the “conventional” procedure, perforated synaptic junctions were treated as single synapses (this treatment could usually be substantiated by viewing the small series of adjacent sections taken for Disector analysis) and synapses on dendritic spines were counted separately from those on shafts. In order to double the efficiency of estimation, the analysis was performed treating each “reference” plane as a “look up” plane and vice versa. (4) Section thickness, which is equivalent to the distance between the “reference” and “look up” planes ( $h_i$ ) was determined by the method of Small (1968). (5) Estimated synapse density (est  $N_v$ ) was then calculated:

$$\text{est } N_v = \frac{\sum Q_i^-}{\sum A_i \times h_i}.$$

**Spine Density Analysis** Light level analysis of dendritic spine density on Golgi-impregnated CA1 pyramidal cells was performed as follows. All slides containing Golgi-impregnated tissue were coded prior to analysis, and the code was not broken until the analysis was complete. In order to be selected for analysis, dendritic segments had to (1) be located between 300 and 500  $\mu\text{m}$  from the pyramidal cell layer in the stratum radiatum, (2) belong to a thoroughly impregnated neuron, (3) remain approximately in one plane of focus, and (4) be greater than 10  $\mu\text{m}$  in length. Appropriate segments were drawn using a camera lucida drawing tube. The number of dendritic spines visible along each segment was counted at 1250 $\times$ . The length of each dendritic segment was then determined from the camera lucida drawings using the SMI morphometry system, and data were expressed as number of spines per 10  $\mu\text{m}$  dendrite. Five dendritic segments per cell and 10 cells per animal were analyzed in this way.

Means for each variable were calculated for each animal and the data were analyzed using unpaired, two-tailed  $t$  tests.

**Table 51.1**

Density of synapses on dendritic spines or shafts obtained using a “conventional” method

Treatment	No. Synapses on Spines/ $\mu\text{m}^3$	No. Synapses on Shafts/ $\mu\text{m}^3$
OVX + O	$0.56 \pm 0.02$	$0.07 \pm 0.01$
OVX + E	$0.90 \pm 0.16^*$	$0.06 \pm 0.01$

Data are from the stratum radiatum of the CA1 region of the hippocampus in ovariectomized (OVX) adult female rats treated with either oil (O) or estradiol (E). Values represent mean  $\pm$  SEM. Data were analyzed with unpaired, two-tailed  $t$  tests,  $n = 3$  in each case.

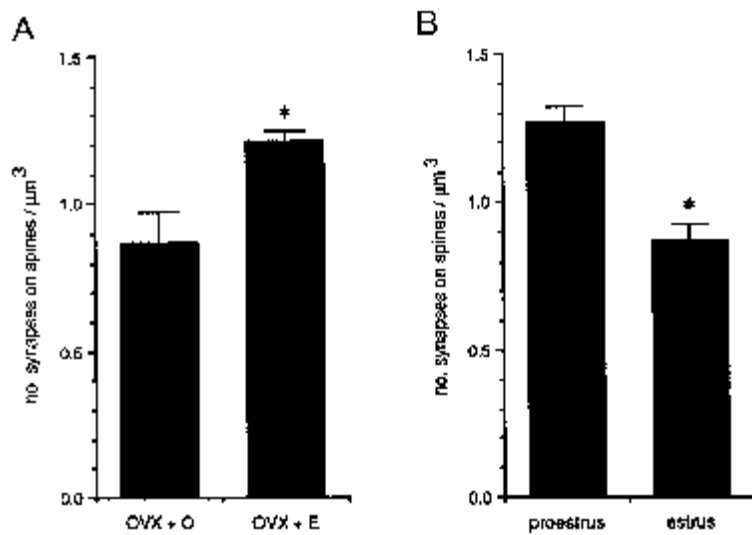
\*Significant difference from OVX + O,  $p < 0.05$ .

## Results

Qualitative examination at the light microscopic level of both Golgi-impregnated and Nissl-stained hippocampal tissue revealed no obvious differences either between estradiol-treated and control animals or animals in the proestrus versus estrus stage of the estrous cycle. Similarly, at the electron microscopic level, no qualitative differences were observed with hormone treatment or between estrous states.

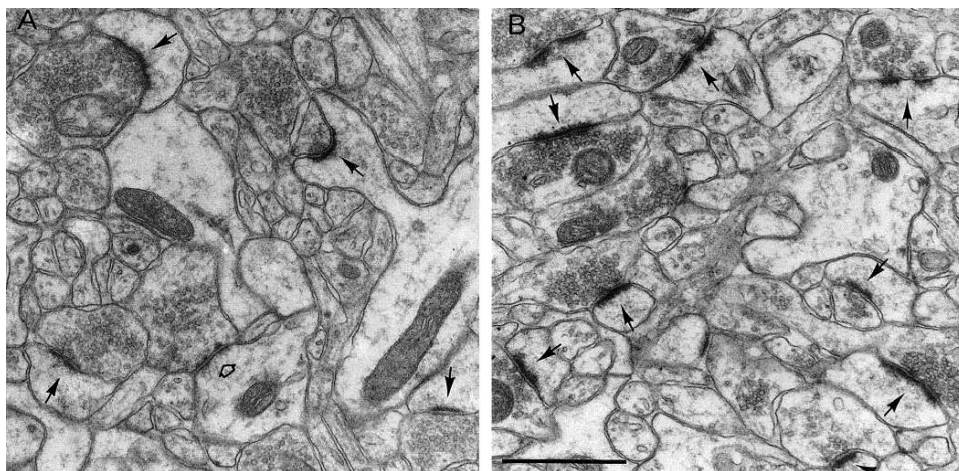
On the other hand, quantitative electron microscopic analysis revealed a selective increase in the density of axospinous synapses in the stratum radiatum both with estradiol treatment and during proestrus when estradiol levels are naturally increased. Analysis using a “conventional” method to estimate hippocampal synapse density in ovariectomized rats revealed a 38% increase in the density of synapses on dendritic spines in estradiol-treated compared to oil-treated animals ( $T = 2.175$ ,  $p < 0.05$ ; table 51.1). On the other hand, estradiol did not appear to affect the density of synapses located on dendritic shafts ( $p > 0.1$ ; table 51.1). Additionally, the mean length of synaptic transects of synapses made on dendritic spines was unchanged by estradiol treatment (mean  $\pm$  SEM): OVX + O,  $0.35 \pm 0.05$   $\mu\text{m}$ ; OVX + E,  $0.27 \pm 0.01$   $\mu\text{m}$  ( $T = 1.637$ ,  $p > 0.1$ ). The results were similar for synapses made on dendritic shafts: OVX + O,  $0.39 \pm 0.04$   $\mu\text{m}$ ; OVX + E,  $0.36 \pm 0.05$   $\mu\text{m}$  ( $T = 0.347$ ,  $p > 0.5$ ).

Analysis using the Disector method confirmed the selective increase in axospinous synapse density with estradiol treatment. Quantitative analysis of hippocampal synapse density in ovariectomized rats that received either estradiol or oil revealed a 28% increase in the density of synapses located on dendritic spines in estradiol-treated compared to oil-treated ovariectomized rats ( $T = 3.154$ ,  $p < 0.025$ ; figures 51.1A, 51.2; table 51.2). As with the “conventional” estimation procedure, estradiol did not appear to affect the density of synapses located on dendritic shafts estimated using the Disector method ( $p > 0.1$ ; table 51.2). Additionally, in agreement with our previously reported



**Figure 51.1**

Estimated density of synapses on dendritic spines in the stratum radiatum of the CA1 region of the hippocampus, in *A*, of an ovariectomized (OVX) rat treated with oil (O) or estradiol (E) or, in *B*, of intact rats in the proestrus or estrus phase of the estrous cycle. Values represent means  $\pm$  SEM obtained using the Disector method. Note that in each case, higher estradiol levels are correlated with a greater density of synapses. Data were analyzed with unpaired, two-tailed *t* tests,  $n = 4$  in each case. \*, significant difference, in *A*, from OVX + O or, in *B*, from proestrus,  $p < 0.025$ .



**Figure 51.2**

Electron micrographs of the stratum radiatum in the hippocampal CA1 region of an ovariectomized adult female rat that received oil (*A*) or estradiol (*B*). Synapses on dendritic spines are marked by *solid arrows*, whereas the *open arrow* in *A* marks a synapse on a dendritic shaft. Scale bar, 1  $\mu\text{m}$ .

**Table 51.2**

Density of dendritic spines and density of synapses on dendritic spines or shafts using the Disector method

Treatment	No. Spines/ 10 $\mu\text{m}$	No. Synapses on Spines/ $\mu\text{m}^3$	No. Synapses on Shafts/ $\mu\text{m}^3$
OVX + O	9.2 $\pm$ 0.6	0.87 $\pm$ 0.10	0.04 $\pm$ 0.03
OVX + E	14.1 $\pm$ 0.8*	1.21 $\pm$ 0.04*	0.06 $\pm$ 0.03
Proestrus	13.3 $\pm$ 0.2	1.27 $\pm$ 0.05	0.04 $\pm$ 0.02
Estrus	10.2 $\pm$ 0.5†	0.87 $\pm$ 0.05†	0.05 $\pm$ 0.02

Data are from the stratum radiatum of the CA1 region of the hippocampus in ovariectomized (OVX) female rats treated with estradiol (E) or oil (O) or in intact adult female rats in the proestrus or estrus stage of the estrous cycle. Values represent means  $\pm$  SEM. Data were analyzed with unpaired, two-tailed *t* tests, *n* = 4 in each case.

\*Significant difference from OVX + O, *p* < 0.025.

†Significant difference from proestrus, *p* < 0.02.

findings (Gould et al., 1990), estradiol treatment of ovariectomized rats resulted in a significant increase of approximately 35% in apical dendritic spine density on Golgi-impregnated CA1 pyramidal cells ( $T = 4.567$ , *p* < 0.02; table 51.2).

When synapse density was analyzed in rats in the proestrus or estrus phase of the estrous cycle, the results were remarkably similar. Quantitative analysis using the Disector method to estimate the density of hippocampal synapses in rats in the proestrus phase, when estradiol levels are highest, or the estrus phase, when estradiol levels are lowest, revealed a 32% decrease in the density of synapses on dendritic spines as estradiol levels drop in the transition from proestrus to estrus ( $T = 3.357$ , *p* < 0.02; figures 51.1B, 51.3; table 51.2). In contrast, no change in the density of synapses on dendritic shafts was observed during the estrous cycle (*p* > 0.1; table 51.2). In parallel to these changes in synapse density and as previously reported (Woolley et al., 1990), apical dendritic spine density on Golgi-impregnated CA1 pyramidal cells decreased by approximately 23% between the proestrus and the estrus phases of the cycle ( $T = 3.412$ , *p* < 0.02; table 51.2). In each of the three experiments, virtually all identified synapses that appeared to be on dendritic spines were asymmetric, while virtually all those observed on dendritic shafts were either clearly symmetric or not easily classified as belonging to either class. This relationship did not appear to be altered by estradiol treatment or during the estrous cycle.

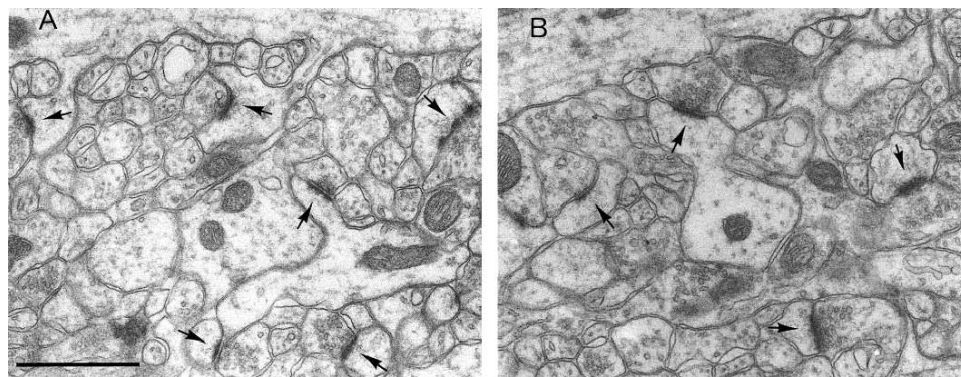
## Discussion

The results presented in this study demonstrate that differences in the density of dendritic spines on hippocampal pyramidal neurons that we have previously observed (Gould et al., 1990; Woolley et al., 1990) reflect an altered pattern of synaptic connectivity between these cells and their afferents. We have observed

a fluctuation in the density of hippocampal synapses occurring during the estrous cycle in experimentally unmanipulated animals. While this fluctuation in synapse density could result from alterations in the levels of either gonadal or pituitary hormones, the fact that we observe such similar, short-term changes with estradiol manipulation strongly suggests that the differences in synapse density we have observed across the estrous cycle are mediated by estradiol.

Previous observations of gonadal steroid regulation of dendritic morphology have been made in sexually dimorphic nuclei that are involved in reproductive behavior (De Voogd and Nottebohm, 1981; Carrer and Aoki, 1982; De Voogd et al., 1985; Kurz et al., 1986; Forger and Breedlove, 1987; Miyakawa and Arai, 1987) or have been associated with the development of reproductive function (Meyer et al., 1978). Of these, the changes that occur naturally in the adult are seasonal (De Voogd et al., 1985; Forger and Breedlove, 1987) and thus take place over long periods of time. The steroid-mediated differences in synapse density we have observed are unique in that they occur naturally, over a period of as little as 24 hr, in a region of the adult mammalian brain that is not known to be sexually dimorphic or involved in reproductive function.

The changes in dendritic spine and synapse density that we have observed are most likely the result of changes in the number of dendritic spine/synaptic junction complexes present in the tissue as a function of hormonal state. While it is possible that treatment effects on synapse size or dendritic arborization could affect estimates of synaptic density without actually changing the number of spine/synapse complexes present, we have evidence that these factors are not responsible for the differences we have observed. First, with regard to synapse size, we found no difference in the mean length of synaptic transects with estradiol treatment; the difference in synapse density calculated with a "conventional" formula was clearly due to a greater number of synaptic profiles observed in estradiol-treated compared to control animals. Further, we observed very similar estradiol-mediated differences in synapse density using the Disector method, which is not biased by differences in the shape, size, or orientation of the test objects subjected to analysis (Sterio, 1984; DeGroot and Bierman, 1986). Second, it seems unlikely that estradiol is affecting dendritic arborization, as we have recently obtained evidence that parameters such as number of apical dendritic branch points and total apical dendritic length in CA1 pyramidal cells are not changed by estradiol treatment (C. S. Woolley and B. S. McEwen, unpublished observations). Thus, while it must be considered a possibility that a change in the volume of some component of the CA1 stratum radiatum could alter the total volume of



**Figure 51.3**

Electron micrographs of the stratum radiatum in the hippocampal CA1 region of an intact adult female rat in the proestrus (A) or estrus (B) stage of the estrous cycle. Synapses on dendritic spines are marked by arrows. Scale bar, 1  $\mu$ m.

this region, making differences in the density of synapses appear as a change in synapse number, it does not appear that an overall shrinking or extension of the pyramidal cell dendritic tree can account for estradiol-mediated changes in dendritic spine and synapse density.

We have observed changes in the density of axospinous synapses, but not in the density of contacts made on dendritic shafts. This, in combination with the fact that we observed very few asymmetric synapses on dendritic shafts in any treatment group, indicates that the decrease in the density of axospinous synapses reflects a loss of spine/synapse complexes with no concomitant increase in the number of synapses made on dendritic shafts. Thus, it appears that, in parallel with fluctuating estradiol levels, we are observing a fluctuation in the actual number of asymmetric synapses present rather than shifts in synapse location between spine and shaft. It is quite likely that transition states between dendritic spines with associated synapses and “empty” dendritic shaft regions exist, for example, presynaptic terminals lacking postsynaptic densities or vice versa. However, it is not possible to identify dendritic shaft regions positively as belonging to a spiny pyramidal neuron or to an aspiny hippocampal interneuron without analyzing dendritic segments reconstructed from serial electron micrographs. Thus, a better understanding of the details concerning the changing relationship between preand postsynaptic elements on pyramidal neurons during fluctuations in dendritic spine and synapse density will depend on continuing studies using serial reconstruction to quantify such incomplete synapses under various hormonal conditions.

It is interesting to note that we did not observe degenerative profiles, either of an electron-lucent or dark variety (Fifkova, 1975), characteristically seen under pathological conditions such as denervation by experimentally induced lesions (Raisman and Mat-

thews, 1972). While it remains a possibility that we might have observed some evidence of degeneration had we chosen other time points for analysis, we feel it is more likely that naturally occurring and reversible changes in connectivity that occur during the estrous cycle are distinct from the patterns of degeneration usually associated with damage.

The mechanism by which these reversible changes in neuronal morphology occur over a period of time as short as 24 hr is unclear. It is possible that regulation of the density of synapses and dendritic spines is a direct effect of estradiol on CA1 pyramidal cells as a small population of these neurons accumulates  $^3\text{H}$ -estradiol (Loy et al., 1988) and a small population has been shown to be physiologically sensitive to estradiol in vitro (Teyler et al., 1980; Wong and Moss, 1991). However, because it is relatively few CA1 pyramidal cells that appear to be directly responsive to estradiol, it seems unlikely that the effects on spine and synapse density we have observed are due to effects of estradiol on CA1 pyramidal cells themselves. On the other hand, because dendritic spines and their associated synapses have been shown to depend on afferent connections (Parnavelas et al., 1974; Caceras and Steward, 1983; Steward and Visant, 1983; Benshalom and White, 1988) and CA1 pyramidal cells receive input from populations of neurons that are estradiol sensitive (Pfaff and Keiner, 1973; Bayer, 1985), it is also possible that differences in spine and synapse density are mediated indirectly by estradiol-sensitive CA1 pyramidal cell afferents. Candidates for estradiol-sensitive inputs to CA1 pyramidal cells include neurons of the medial septum/diagonal band, entorhinal cortex, locus ceruleus, and raphe nuclei. The neuroanatomical basis for this hypothesis is currently being investigated in our laboratory. Another intriguing hypothesis is suggested by the recent finding of Blaustein et al. (1991) that estrogen receptors can be localized to axonal terminals in the guinea pig hypothalamus by combined electron

microscopy/immunocytochemistry. It is not yet known whether a similar receptor distribution exists in the hippocampus of the rat. Although the possibility is highly speculative at this point, estrogen-sensitive terminals making synapses on dendritic spines of CA1 pyramidal cells could provide an anatomical basis for indirect, local action of estradiol in the regulation of dendritic spine and synapse density in this neural region.

As synapses located on dendritic spines are the sites of the majority of excitatory input to CA1 pyramidal neurons (Westrum and Blackstad, 1962; Harris and Stevens, 1989), our findings strongly suggest that activity of CA1 pyramidal cells could be modulated by circulating levels of estradiol. The fact that we have observed changes specifically in asymmetric synapses, which are presumably excitatory (Westrum and Blackstad, 1962; Andersen, 1987; Harris and Stevens, 1989), supports the possibility that CA1 pyramidal cell excitability could be increased when estradiol levels are high, at least in part, as a result of an increase in the density of excitatory inputs they receive. Our findings may help to explain previously reported effects of ovarian steroids on hippocampal pyramidal cell physiology in vivo that have correlated high levels of steroids with increased hippocampal neuronal activity both in the case of hormonal manipulation and during the estrous cycle (Terasawa and Timeras, 1968; Kawakami et al., 1970).

Furthermore, it seems likely that cognitive functions, which are undoubtedly dependent upon synaptic communication between neurons, would be affected by differences in synapse density and thus may be subject to fluctuations in hormone levels. The fact that we observe differences in hippocampal synapse density as great as 30% implies that normally occurring differences in the circulating level of estradiol might have important consequences for hippocampal function. As cognitive processes, particularly learning ability, known to involve the hippocampus (O'Keefe and Nadel, 1978; Olton, 1983; Squire, 1983), also appear to be influenced by estradiol both developmentally (Williams and Meck, 1991) and in the adult (Diaz-Veliz et al., 1989; Hampson, 1990), it is interesting to speculate on the possibility that estradiol could affect these processes, at least in part, through alterations in synapse density.

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## Introduction

Evidence has been gathered for the existence of a hypothalamic prolactin (PRL) system. Immunohistochemical studies have demonstrated immunoreactive PRL in the hypothalamus (12, 13) and other areas of the adult rat brain (26). The detection of PRL-mRNA (24) in hypothalamic cells and the presence of PRL protein (6, 10) in the hypothalamus of hypophysectomized animals suggests that PRL is of local and not of pituitary origin. PRL may indeed be a neuropeptide that modulates reproductive behavior. PRL has been shown to inhibit lordosis and to promote the onset of maternal behavior (5, 9). These behavioral effects might be mediated via specific binding sites in neural tissue (8) and intracellular second messenger-systems coupled to PRL receptors (17). It appears to concur with a role in controlling gender-specific behavior that there are clues to a sexual differentiation of the hypothalamic PRL system. Recently, De Vito (6) reported differences in PRL-immunoreactivity between male and female adult rat hypothalamus. Higher PRL levels as quantified by radioimmunoassay were found in females than in males. Hypophysectomy decreased the PRL content in the female but not in the male hypothalamus (6), and estradiol treatment selectively restored PRL levels in hypophysectomized females (7). In addition, female hypothalamic tissue is characterized by a higher density of PRL binding sites in comparison to male hypothalamus (21).

It is generally believed that sexual differentiation of the rat brain is brought about by perinatal exposure to  $17\beta$ -estradiol locally aromatized from gonadal testosterone during a "critical" period (15, 20). The onset of the critical period coincides with the surge of systemic testosterone in the male rat fetus on E 18 (27). However, this concept has recently been contested because investigations on the sexual differentiation of dopaminergic neurons in vitro have shown that the development of sexual dimorphisms may be initiated well before that time and may proceed in the absence of gonadal hormones (23). The purpose of the present study was to investigate whether sex differences of

hypothalamic PRL systems, too, are determined before the onset of the critical period of sexual differentiation of the brain or depend on the prenatal surge of testosterone occurring at E 18.

## Materials and Methods

Sprague–Dawley rats were mated during a 12-h interval from 10 p.m. to 10 a.m. (10 p.m. was considered to be the beginning of E 0). Embryos removed in the morning of E 14 or E 17 had a crown-rump length of 10.5–11.0 mm and 16–18 mm, respectively. Male and female embryos were separated by inspection of the gonadal anlage (11, 22). Care was taken to leave the hypophyseal anlage in situ when removing the brains. Culturing techniques have been described previously in detail (4, 18). Briefly, the diencephalon was dissected, enzymatically and mechanically dissociated and filtered through nylon mesh (pore size 30  $\mu$ m). Cells were plated at a density of  $2.5 \times 10^5$  cells/cm<sup>2</sup> on poly-L-lysine (1  $\mu$ g/ml)-coated plastic culture dishes. Cultures were raised in a water-saturated atmosphere of 5% CO<sub>2</sub> and 95% air with Phenol red-free MEM Earle medium supplemented with 10% castrated horse serum, in which  $17\beta$ -estradiol and testosterone were not detectable by radioimmunoassay. Cultivation was terminated after 10 days in vitro (DIV). After fixation with 4% paraformaldehyde, immunocytochemistry to PRL was performed using a highly specific polyclonal antiserum (1:500, NHPP, USA) and the ABC method (14). Antigen–antibody complexes were visualized by diaminobenzidine reaction in the presence of 0.01% H<sub>2</sub>O<sub>2</sub>. Controls were obtained by replacing PRL antiserum with normal rabbit serum or by liquid phase preabsorption of PRL antiserum with rat PRL (purity > 99.5%; NHPP, USA), 5 ml antiserum (1:500) were incubated with 1 mg PRL at pH 7.2 and room temperature for 4 hours.

For quantitative Western blots, proteins were separated into an insoluble and a soluble fraction by lysis in a buffer containing 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 110 mM NaCl, 50 mM EDTA, and 0.5% (v/v) Triton X-100 (pH 7.2). After centrifugation (15 min, 12,000 *g*<sub>av</sub>,

4°C), supernatant and pellet were dissolved in 62.5 mM Tris-HCl, 2 mM EDTA, 2% (w/v) SDS, and 5% mercaptoethanol (pH 6.8), heated to 100°C for 5 min, pooled and stored at -20°C. SDS-PAGE was carried out under reducing conditions on 12.5% polyacrylamide gels with a sample volume of 10 µl. After electrophoresis, proteins were transferred to nitrocellulose and incubated overnight with anti-PRL (1:2,000 in TBS-Tween, pH 7.6, 4°C). The immunoreaction was visualized and the controls carried out as described above for immunocytochemistry. Densitometric evaluation of immunoreactive bands was performed with a video-adapted image analysis program (Image-Pro, Media Cybernetics, USA). A standard curve was prepared with rat PRL (NHPP, USA). Grey level values were linear between 500 pg and 100 ng of PRL. Amounts of PRL extracted from cultures were in the range of 5 to 10 ng. The protein content of the cultures was determined following the procedure of Smith et al. (25).

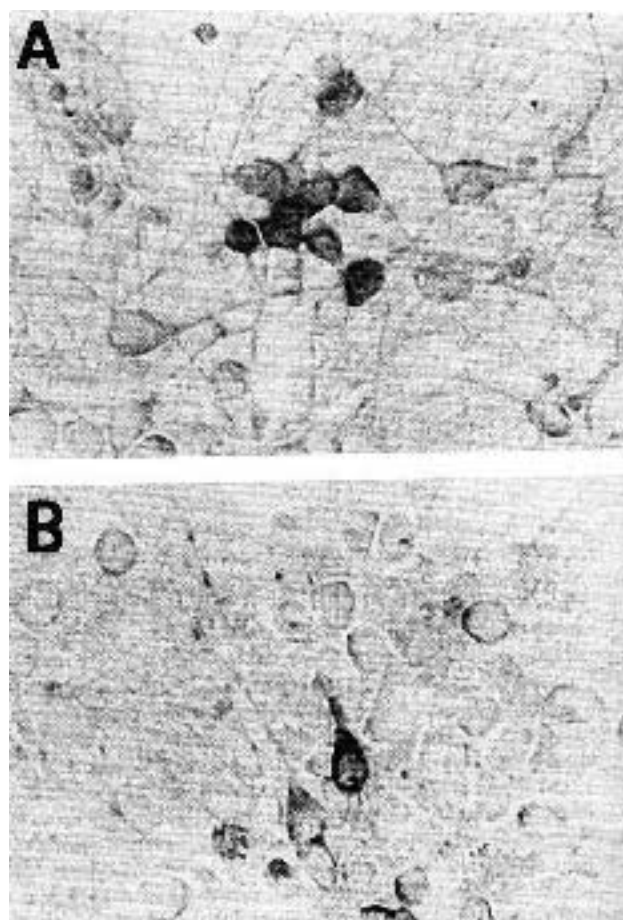
Gender-specific cultures were raised from 4 litters of E 14 and 4 litters of E 17. Material from one litter was sufficient to prepare, per gender, 4–5 cultures (in 3.5-cm culture dishes) for Western blots and 4–5 cultures (in 24-well plates) for immunocytochemistry. Immunoreactive perikarya were counted by screening entire wells. Data obtained from each litter were subject to an analysis of variances (Scheffé test). Both data from male and female cells were normally distributed. Sex differences were statistically significant within each litter. Therefore, data were pooled and means calculated per gender and age group. The pooled data were analyzed with the *t*-test.

## Results

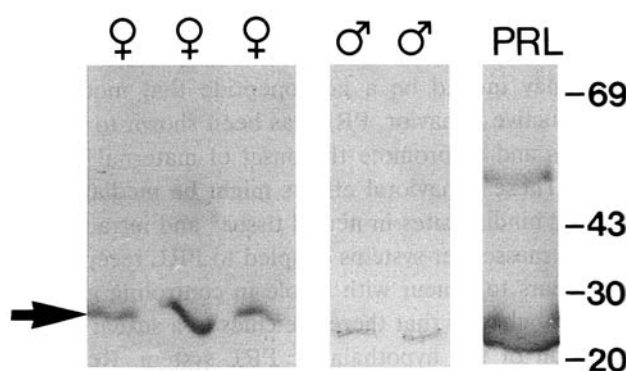
A distinct population of cells immunoreactive to PRL (figure 52.1) was present both in cultures prepared from E 14 and E 17 donors. No such cells were seen in control preparations. Higher numbers of PRL-IR cells were consistently found in female than in male cultures (figure 52.3). Western blots of protein extracts from the cultures showed a heavily stained band with a  $M_r$  of about 23–25 kDa with an electrophoretic mobility identical to the rat PRL reference standard (figure 52.2). Again, both in extracts from E 14 and E 17 cultures, heavier immunoreactive bands were seen with female than with male donors (figure 52.2). Densitometric evaluation of immunoblots gave about three times (E 17) and two times (E 14) higher PRL amounts in female than in male cultures (figure 52.3).

## Discussion

This in vitro study demonstrates that sex differences in numbers of hypothalamic PRL-IR cells and in



**Figure 52.1**  
PRL-immunoreactive cells in female (A) and male (B) E 14 diencephalic cultures after 10 days in vitro. The immunostained cells are surrounded by unstained neurons and glia. (×400)



**Figure 52.2**  
Western blots of PRL extracted from male and female E 17 cultures after 10 days in vitro. The right-hand lane shows an immunoblot of a PRL standard (50 ng/10 µl injection volume) resulting in a heavily stained band corresponding to an  $M_r$  of about 23–25 kDa. Immunostained bands with the same mobility are present in the culture extracts and are more conspicuous in female than in male tissue. The nature of the immunostained band with an  $M_r$  of about 50–60 kDa seen in the standard immunoblot is not clear.

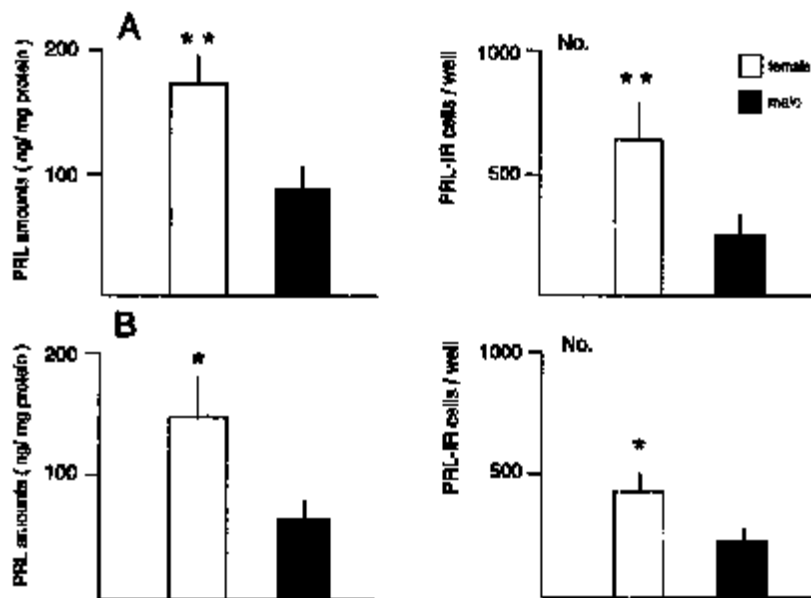


Figure 52.3

Amounts of PRL extracted from and numbers of PRL-immunoreactive cells in E 17 (A) and E 14 (B) diencephalic cultures after 10 days in vitro. Each value gives the mean  $\pm$  S.D. from 16–20 cultures. \*\*  $P \leq 0.01$  female vs. male E 17; \*  $P \leq 0.05$  female vs. male E 14.

amounts of PRL develop in dissociated cell cultures of embryonic rat diencephalon. Since male and female neurons contain about the same amount of PRL per cell, it is the size of this specific subpopulation of neurons and not the expression of PRL in the individual neuron that is affected by the process of sexual differentiation. Sex differences in neuron numbers are a characteristic of many sexually dimorphic brain nuclei (16). The sex differences in numbers of hypothalamic PRL cells may be due to enhanced proliferation of precursor cells taking place in the female before removal of the tissue, to a more rapid differentiation of female PRL cells, and/or to a higher survival rate of cultured neurons of female donors. The results confirm previous observations of sexual dimorphisms of the hypothalamic PRL system in the adult rat (6, 7) and suggest that this is due to a prenatally occurring process of sexual differentiation of a certain subpopulation of hypothalamic neurons. The salient point of the present results is that sexual dimorphisms developed in cultured cells removed from the embryo as early as E 14 or E 17, respectively, and grown in the absence of sex steroids. It can therefore be excluded that sexual differentiation of these cells is dependent upon sex differences in systemic testosterone levels, which do not evolve prior to E 18 (2, 27). This is in contradiction to the generally accepted theory which holds that sexual differentiation of the brain is caused by the organizational effect of gonadal steroids present during a critical period of brain development (1, 20). We have previously published a number of results with which

we have questioned the general validity of this concept (see Ref. 23 for review). Similarly to the present approach, morphological and functional sex differences of catecholaminergic neurons were found to develop in cultures of embryonic rat brain tissue removed at E 14 and raised in the absence of detectable amounts of sex steroids (4, 11, 18, 19, 22). Such sexual dimorphisms even developed when the mothers were treated with antisteroids on E 12 and E 13 in order to definitely exclude steroid-dependent organizational events that may occur in utero before the tissue is taken into culture (3). We feel thus safe to conclude that epigenetic control by the hormonal environment cannot be the only mechanism responsible for sexual differentiation of hypothalamic PRL neurons. Factors other than gonadal steroids, such as cell-autonomous realization of a sex-specific genetic program must be invoked to explain the generation of sex differences. We propose that a cascade of cell-intrinsic and -extrinsic (epigenetic) events is needed to establish a fully developed male or female brain.

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All sex differences in mammalian and avian development originate ultimately from the action of genes located on the sex chromosomes. For example, the Y-linked gene *Sry* directs the sexual differentiation of the mammalian gonad by committing that tissue to a testicular rather than ovarian pattern of development (Goodfellow and Lovell-Badge, 1993). Once testes or ovaries develop, however, differences in their secretions induce sexual differentiation of non-gonadal tissues (Jost, 1958), including the brain. Testosterone, or its metabolites such as estradiol, act on the brain during critical periods of development to induce masculine patterns of neural differentiation that lead to sex differences in brain and behavior (Phoenix et al., 1959; Goy and McEwen, 1980; Arnold and Gorski, 1984).

In some cases, however, sexual dimorphisms in non-gonadal tissues are difficult or impossible to explain as the result of gonadal steroid action. (1) In several mammalian species, male embryos develop faster than female embryos (Erickson, 1997) before differentiation of the gonads. X- and Y-linked genes contribute to this sex difference (Burgoyne et al., 1995). (2) In the tammar wallaby, several sexually dimorphic structures, such as pouch and scrotum, differentiate before the gonads have differentiated (Renfree and Short, 1988). (3) Neurons dissociated from embryonic male or female rat brain cultured under identical conditions nevertheless grow in a sexually dimorphic manner, with female cultures developing more tyrosine hydroxylase (TH) or prolactin-immunoreactive neurons, although the cells are harvested before the onset of sexually dimorphic gonadal secretions (Beyer et al., 1991, 1992a,b). (4) Numerous X-linked genes are expressed differently in males and females and can lead to sex differences in traits. In New World primates, for example, females express more X-linked photopigment alleles than males, which generates a sex difference in retinal photosensitivity (Jacobs, 1998; Dulai et al., 1999). (5) In zebra finches, genetic females that possess testes develop a feminine neural circuit for song (Wade and Arnold, 1996), and it has so far proved difficult to prevent masculine neural differentiation by manipulat-

ing gonadal steroid action in genetic males (Arnold, 1997).

Comparisons of behavioral phenotypes across mouse strains have revealed that the Y chromosome encodes genes that influence specific sexually dimorphic behaviors or neural phenotypes (Maxson, 1992, 1996a,b; Guillot et al., 1995; Hensbroek et al., 1995; Sluyter et al., 1995, 1996). Y chromosome dosage also appears to have significant behavioral effects in humans (Ratcliffe et al., 1990). These studies support a role for the Y chromosome in the development of male neural and behavioral phenotypes.

In the present paper we introduce a powerful mouse model system for testing the idea that X- or Y-linked genes contribute directly to sexual differentiation of the brain via nonhormonal mechanisms. Our test involves comparisons of the brain and behavior of mice that have the same gonads but possess different complements of sex chromosomes. We identify one sexual dimorphism in brain, the density of vasopressin (VP)-immunoreactive (ir) fibers in the lateral septum, which is more masculine in mice with XY sex chromosomes than in mice with XX chromosomes. The difference is likely a direct effect of genes encoded on the sex chromosomes.

## Materials and Methods

### Mouse Stocks

All mice were random-bred MF1 except for the Y chromosome, which derived from strain 129 (129/SvEv-*Gpi1*<sup>c</sup> Y) (Simpson et al., 1999). Some mice possessed a variant of Y<sup>129</sup> deleted for the testis-determining gene *Sry* [*Tdy*<sup>m1</sup> mutation of Lovell-Badge and Robertson (1990); Gubbay et al. (1992)], here designated Y<sup>−</sup>. Thus, XY<sup>−</sup> mice are female (defined by the presence of ovaries). In some mice an *Sry* transgene was inserted into an autosome, creating XY<sup>−</sup>*Sry* mice that possess testes and are fully fertile males (Mahadevaiah et al., 1998). XY<sup>−</sup>*Sry* males were bred with normal MF1 XX females to produce four genotypes: XX females, XY<sup>−</sup> females, XY<sup>−</sup>*Sry* males, and XX*Sry*

males. Comparison of these four groups (using two-way ANOVAs; see below) allowed assessment of the independent effects of sex (male vs female) and sex chromosome complement ( $XY^-$  vs  $XX$ ) and their interaction. All four genotypes occur in the same litters so that prenatal and postnatal environment and litter effects are distributed across groups. In addition,  $XY^{129}$  males (MF1 males of the same strain as the other mice except that the  $Y^{129}$  chromosome was not deleted for *Sry*) were compared with  $XY^-Sry$  males to test for any differences attributable to the different *Sry* genes (transgenic vs endogenous).

### Experiment 1

Mice were bred at the Medical Research Council National Institute for Medical Research. Genotype was determined by PCR analysis of the presence of the Y long-arm gene family *Ssty* and of the *Sry* transgene at the time of weaning and again after behavioral analysis. As adults (7–8 weeks of age) the mice were shipped to the University of Virginia (Charlottesville, VA), where they were tested for aggression, copulatory behavior, and social exploration. The results on aggressive behavior will be presented in a separate publication. The mice were anesthetized and perfused with fixative (see below). Fixed brains and spinal cords were shipped to other laboratories for histological analysis of several sexually dimorphic neural systems, including the vasopressin (VP) innervation of the lateral septum, tyrosine hydroxylase (TH)-immunoreactive (ir) neurons of the anteroventral periventricular nucleus of the preoptic region (AVPV), and the spinal nucleus of the bulbocavernosus (SNB), as described below. All handling of mice and analyses were conducted by investigators who were unaware of the genotypes of the mice. At the University of Virginia, males were housed individually. Females were housed in groups of two to three for 2 months until they were treated as described below and housed individually. Mice were housed on a 12 hr light/dark cycle (lights off at 1:00 P.M. EDT) and received ad libitum access to food (Purina mouse chow 5001) and water.

**Behavioral Tests: Surgery, Hormone Treatment, and Social Exposure** Mice used as experimental subjects were gonadectomized bilaterally. Testes were collected and weighed for later indirect check of genotype ( $XXSry$  males have smaller testes than  $XY^-Sry$  males). Surgery was conducted under ketamine/xylazine anesthesia (xylazine, 100 mg/kg; ketamine, 10 mg/kg). At the time of surgery each mouse received a subcutaneous SILASTIC capsule [1.02 mm inner diameter (id)  $\times$  2.16 mm outer diameter (od)] in the midscapular region filled with 7 mm of testosterone. For the be-

havior tests, the group sizes were  $XY = 10$ ,  $XY^-Sry = 17$ ,  $XXSry = 15$ ,  $XY^- = 19$ , and  $XX = 17$ .

**Stimulus Animals** One week after surgery each animal was given exposure to other mice 3–5 hr before the dark cycle (Wersinger et al., 1997). Gonad-intact C57BL/6J males served as stimulus animals for both social exposure and social exploration tests. For the social exposure and sex behavior tests, female C57BL/6J adults were ovariectomized and received a subcutaneous implant of estradiol benzoate dissolved in sesame oil (50  $\mu$ g in 0.025 ml) infused into a SILASTIC implant (1.98 mm id  $\times$  3.17 mm od) sealed with SILASTIC adhesive. The females received a subcutaneous injection of progesterone (P) [100  $\mu$ g in 0.025 ml sesame oil; method of Rissman et al. (1997)] 2–5 hr before each sex test. These same females were also used for social exploration tests, in which no P injections were given.

**Masculine Mating Tests** Starting 3 weeks after gonadectomy, each mouse was tested for masculine sexual behavior beginning 2–3 hr after the start of the dark cycle according to the procedure of Wersinger and Rissman (2000a). Briefly, tests were conducted in the dark in clear Plexiglas cages (18  $\times$  38 cm) placed on a mirror stand to allow ventral viewing and permit the observer to distinguish between mounts with or without intromission. The tests lasted for 30 min, or until the test animal ejaculated. If the pair was engaged in mounting at the end of 30 min, the test was continued until either the test animal ejaculated or the pair stopped interacting for 5 min. During the tests, the latencies and numbers of each attempted mount, mounts with thrusts, mounts with intromission, and ejaculation were recorded as were the numbers of thrusts and intromissions per mount. For data analysis of behavioral latencies and frequencies, only tests that included the behavior of interest were scored.

**Social Exploration Tests** Social exploration was tested 1 week after the sexual behavior test 2–3 hr after the start of the dark cycle, using a procedure described in Wersinger and Rissman (2000b). A Plexiglas test box was divided into three areas. Anesthetized stimulus mice were placed in each of the two end chambers, an intact male at one end and an ovariectomized estrogen-implanted female at the other end. The number of visits to each stimulus animal, the total amount of time spent with stimulus animals, and the amount of time each mouse spent sniffing each stimulus animal were recorded (Wersinger and Rissman, 2000b). The social exploration test is sensitive to central processing of social stimuli, but also to individual differences

in basic sensory processing, for example in olfactory sensitivity.

**Tissue Collection** At the end of behavioral testing the mice were anesthetized with sodium pentobarbital. Blood was collected for testosterone radioimmunoassay. The mice were perfused briefly with 0.9% saline followed by 5% acrolein in 0.1 M sodium phosphate buffer (PB), pH 7.6. The bodies of the mice were sent to the University of California Los Angeles for histological analysis of the SNB. Fixed brains were placed in a solution of 30% sucrose in PB and shipped to the University of Massachusetts (Amherst, MA). Brains were blocked into 2- to 4-mm-thick transverse slices. The slices that included the septum and the AVPV were split into a dorsal and ventral half by a horizontal cut at the level of the crossing of the anterior commissure. The portion containing the AVPV was frozen and shipped to the Oregon Regional Primate Research Center (Beaverton, OR) for analysis of TH immunoreactivity in the AVPV. The other samples were stored in PB-buffered sucrose solution at 4°C until they were sectioned transversely at 35  $\mu$ m with a freezing microtome for analysis of VP immunoreactivity in the lateral septum.

**Testosterone Radioimmunoassay** Testosterone levels were determined by radioimmunoassay conducted by the University of Virginia Core Ligand and Assay Laboratory (Charlottesville, VA). Samples were run in duplicate in a single assay. The range of the assay was from 0.1 to 25.0 ng/ml. The average intra-assay coefficient of variability was 10.8%. Two animals were excluded from further analysis because they possessed exceptionally high levels of testosterone at the time they were killed.

**Vasopressin Immunocytochemistry** Sections were processed for VP immunoreactivity at room temperature unless stated otherwise. VP immunoreactivity was located with rabbit anti-VP serum (ICN Laboratories, Costa Mesa, CA) in a 1:4000 dilution for 90 min at 37°C, followed by detection of the primary antibody by biotinylated goat anti-rabbit serum (Vector Laboratories, Burlingame, CA) and the avidin-biotin complex ABC detection system (Vector Elite Kit, Vector Laboratories) followed by visualization of the antibody complex using nickel-intensified DAB as the chromogen as described in Villalba et al. (1999). Microscopic images were captured under bright-field illumination using a CCD camera linked to a computer. The density of VP-ir fibers in the lateral septum was examined in the section that contained the highest fiber density in these areas [corresponding to figure 30 in the atlas

of Paxinos and Franklin (1998)]. Fiber density was analyzed by computerized gray-level thresholding using the NIH Image software. The light intensity and camera setting were kept constant across the sections to standardize measurements. Fiber density was expressed as the number of pixels covered by VP-ir fibers in an image of a 0.25-mm-square sampling area immediately bordering the ventricular wall. Group sizes were XY = 10, XY<sup>-</sup>Sry = 17, XXSry = 13, XY<sup>-</sup> = 15, and XX = 13.

**Tyrosine Hydroxylase Histochemistry of the AVPV** To determine the number of dopaminergic neurons in the AVPV of each animal, 20- $\mu$ m-thick frozen sections through the preoptic region were cut on a sliding microtome and collected in chilled potassium PBS. Dopaminergic neurons were labeled by incubating tissue sections at 4°C for 72 hr in a 1:1000 dilution of an antiserum directed against TH (EugeneTech, Allendale, NJ), which was localized with an affinity-purified goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (BioSource International, Camarillo, CA) as described in detail previously (Sawchenko and Swanson, 1981). The sections were mounted, counterstained with ethidium bromide for cytoarchitectonic orientation (Schmued et al., 1982), and coverslipped with buffered glycerol. The number of TH-ir neurons within the AVPV was counted in every third section using fluorescence microscopy and then corrected with Abercrombie's method (Abercrombie, 1946). The counted objects did not differ in size, and the section thickness did not vary between experimental groups; thus the results provide estimates of the relative number of TH-ir neurons in different groups, not absolute cell numbers. The group sizes were XY = 8, XY<sup>-</sup>Sry = 11, XXSry = 13, XY<sup>-</sup> = 16, and XX = 14.

**Spinal Nucleus of the Bulbocavernosus** Motoneurons were discriminated from non-motoneurons in the region of the SNB using immunostaining for Islet-1, a motoneuronal marker (Ericson et al., 1992, 1996). The lumbosacral region of spinal cords was immersed in 30% sucrose in 0.1 M PB and then frozen-sectioned horizontally on a sliding microtome at 40  $\mu$ m and processed for Islet-1-like immunoreactivity at room temperature. After three rinses in 0.05 M Tris-buffered saline (TBS), sections were incubated in 1% sodium borohydride (in 0.1 M PB). Sections were then incubated in TX100 solution (0.05 M TBS with 0.3% Triton X-100) with 4% normal horse serum (NHS) and 1% hydrogen peroxide for 30 min and then reacted with mouse anti-Islet-1 antibody (39.4D5, Developmental Studies Hybridoma Bank, Department of Biological

Sciences, University of Iowa, Iowa City, IA) at a concentration of 1:80 in TX100 solution with 2% NHS for 60 min. Sections were then incubated with the secondary antibody (horse anti-mouse IgG; Vector Elite Kit, Vector Laboratories) in TX100 solution with 2% NHS for 60 min and reacted with ABC reagent, and then 0.05 M TBS with 0.05% DAB and 0.003% H<sub>2</sub>O<sub>2</sub>. Some spinal sections were processed without the primary or secondary antibody, in which case no Islet-1-like staining was observed.

SNB motoneurons were counted within eight consecutive sections comprising a 320- $\mu$ m-thick horizontal slab with as most dorsal limit the section that contained the most dorsal Islet-1-ir cells ventral to the central canal. Cells were counted only if they expressed Islet-1-immunoreactivity and were located within 175  $\mu$ m of the midline and were between the lumbar enlargement and the level of the sacral region at which the spinal cord width was >1.2 mm. In addition, nuclear size and diameter of 20 Islet-1-positive neurons was measured by using NIH Image morphometric software. Abercrombie's correction was used to correct neuron number (Abercrombie, 1946). The group sizes were XY = 9, XY<sup>-</sup>Sry = 9, XXSry = 8, XY<sup>-</sup> = 9, and XX = 9.

**Statistical Tests** All data were analyzed by ANOVAs followed by planned comparisons (Tukey–Kramer tests) to test for differences between pairs of groups. Some of the behavioral data were analyzed by ANOVA on ranks if they failed to meet criteria for a normal distribution. For all analyses except that of VP-ir fibers in the lateral septum (see experiment 2), two basic ANOVAs were run. The first was a two-way ANOVA on the four groups of mice that were progeny of XY<sup>-</sup>Sry fathers and XX mothers, with two factors of sex chromosome complement (XY<sup>-</sup> vs XX) and sex (male vs female or Sry vs no Sry). This analysis allowed us to determine whether there was a main effect of sex that would indicate a phenotypic difference correlating with the presence of testes or ovaries, or a main effect of sex chromosome complement (XY<sup>-</sup> vs XX), or an interaction. The predictions of the present paper were that (1) there would be a main effect of sex chromosomes or an interaction of sex chromosomes and sex, or both, and (2) planned comparisons would show a difference between XY<sup>-</sup>Sry males and XXSry males, or between XY<sup>-</sup> females and XX females, or both. The second analysis was a one-way ANOVA comparing two male groups (XY vs XY<sup>-</sup>Sry), which tests whether the Sry transgene has a different effect than the endogenous Sry on the dependent variables measured. Our hypothesis was that there would be no difference in that analysis. When we found a significant difference between male groups, we conducted a fur-

ther nested ANOVA with litter as the nested variable. This analysis determined whether within-group differences among litters could be eliminated as a significant contributor to the between-group difference and whether the difference was robust enough to survive the loss of power that is inherent in the nested analysis. The  $\alpha$  level for all tests was 0.05.

## Experiment 2

A second experiment was run to replicate the finding in the first experiment that there was a significant effect of sex chromosome complement on VP-ir fiber density in the lateral septum. Mice of the same genotypes as in experiment 1 were bred at National Institute for Medical Research (NIMR; Mill Hill, London, UK) and then shipped to the University of Massachusetts. There they were gonadectomized and implanted with SILASTIC capsules filled with T as in experiment 1. Three weeks later, the mice were killed and processed for VP immunoreactivity as in experiment 1. The tissue of one XXSry male was excluded from analysis because of excessively high background staining. The statistical analysis of VP-ir paralleled that for other dependent variables, except that experiment number was included as a third factor and data were standardized per experiment as  $z$ -scores ( $z = (x - m)/s$  where  $m$  = group mean and  $s$  = standard deviation). Thus, we conducted a three-way ANOVA on the XY<sup>-</sup> versus XX ( $\pm$ Sry) genotypes [three factors were sex chromosomes (XX vs XY<sup>-</sup>), sex (male vs female), and experiment number]. A second, two-way ANOVA was used to compare XY and XY<sup>-</sup>Sry groups in the two experiments [factors were Sry (endogenous Sry in XY vs Sry transgene in XY<sup>-</sup>Sry) and experiment number]. Group sizes were XY = 7, XY<sup>-</sup>Sry = 7, XXSry = 8, XY<sup>-</sup> = 10, and XX = 10.

## Experiment 3

The first two experiments showed opposite differences between XY<sup>-</sup> females and XX females in the density of VP-ir fibers in the lateral septum. To test the hypothesis that this inconsistency was the result of variability induced by uncontrolled exposure of fetal females to androgens originating from adjacent males in utero (vom Saal and Bronson, 1978), litters of mice were produced containing only females. XY<sup>-</sup>Yq-del<sup>RIII</sup> males (Mahadevaiah et al., 2000) were mated to MF1 females. The Yq-del chromosome is of RIII strain origin and carries a deletion of Yq (Conway et al., 1994). Although initially sterile, older males become fertile because of random loss of one or the other Y in a proportion of spermatogonia. Most of these males produce only female offspring (XX and XY<sup>-</sup> genetically identical to those in experiments 1–3), apparently because of selection against Yq-del sperm in the female

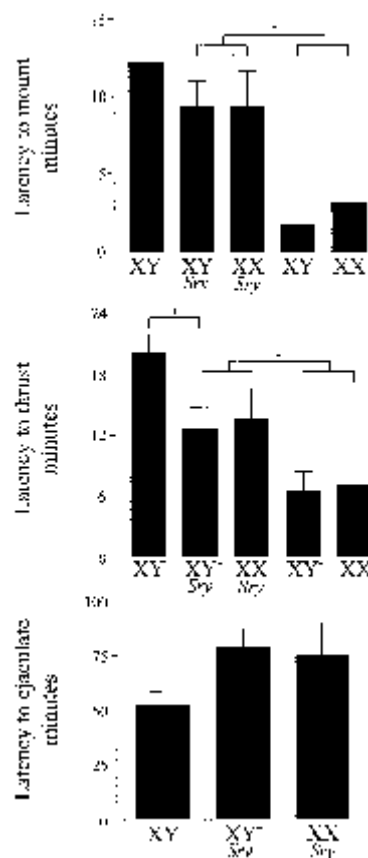
tract (P. S. Burgoyne, unpublished results). Mice were bred at NIMR. In experiment 3A they were shipped to the University of Virginia where they underwent surgery, hormone implantation, and behavioral testing similar to that described for experiment 1 (data not shown), after which fixed brains were shipped to the University of Massachusetts for analysis of septal VP-ir fiber density. In experiment 3B the mice were shipped directly to the University of Massachusetts, where they were treated as in experiment 2 before measuring septal VP-ir fiber density. A two-way ANOVA was used to compare XX and XY<sup>-</sup> groups in the two experiments (factors were sex chromosome complement, XX vs XY<sup>-</sup>, and experiment number). The group sizes were XX = 7 and 16 and XY<sup>-</sup> = 7 and 17 in experiments 3A and 3B, respectively.

## Results

### Experiment 1

**Testosterone Levels** At the time the mice were killed, plasma testosterone levels were not statistically different among the groups because they all had received identical treatment with testosterone during testing: XY males ( $2.17 \pm 0.27$  ng/ml; mean  $\pm$  SEM), XY<sup>-</sup>Sry males ( $2.21 \pm 0.13$ ), XXSry males ( $1.81 \pm 0.13$ ), XY<sup>-</sup>females ( $3.01 \pm 0.61$ ), and XX females ( $3.68 \pm 1.27$ ). The slightly higher but not significantly different levels in females, if anything, would tend to diminish any sex differences in dependent variables that are sensitive to both adult and perinatal circulating levels of testosterone, such as the density of VP-ir fibers in the lateral septum. It should not influence, however, phenotypes that are insensitive to the level of testosterone in adulthood, for example, the numbers of neurons in the SNB or AVPV.

**Masculine Sexual Behavior** In analyses of specific behaviors we included only data from mice that displayed the behavior. Female mice had shorter latencies to mount than did males and also shorter latencies to mount with thrusts (figure 53.1). There was a main effect of sex on latency to mount ( $F_{(1,59)} = 19.42$ ;  $p < 0.00005$ ) but no significant effect of sex chromosomes or a significant interaction. The same pattern was found for latency to thrust: a main effect of sex ( $F_{(1,45)} = 6.02$ ;  $p < 0.02$ ) (figure 53.1), but no significant effect of sex chromosomes or a significant interaction. Mount latencies were not different between XY and XY<sup>-</sup>Sry males, but XY<sup>-</sup>Sry males began thrusting sooner than XY males ( $F_{(1,21)} = 6.05$ ;  $p < 0.025$ ). The latter difference was also significant when a nested ANOVA was performed ( $F_{(1,21)} = 6.11$ ;  $p < 0.04$ ). Although XY males began thrusting later than XY<sup>-</sup>Sry



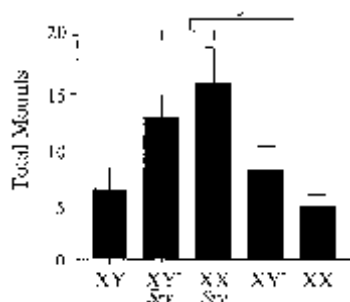
**Figure 53.1**

Latencies to mount, thrust, and ejaculate during sex behavior tests (means  $\pm$  SEM). Within the four genotypes generated in the core cross, males (XY<sup>-</sup>Sry and XXSry) had longer latencies to mount and to thrust than did females (XY<sup>-</sup> and XX) ( $p < 0.00005$ ). XY males, which derived from a different cross, had longer latencies to mount than XY<sup>-</sup>Sry males. Group sizes *left to right* were as follows: 8, 15, 16, 15, and 13 for latency to mount; 7, 14, 14, 10, 7 for latency to thrust; 5, 11, and 10 for latency to ejaculate.

males, they tended to ejaculate sooner ( $F_{(1,16)} = 4.31$ ;  $p = 0.057$ ).

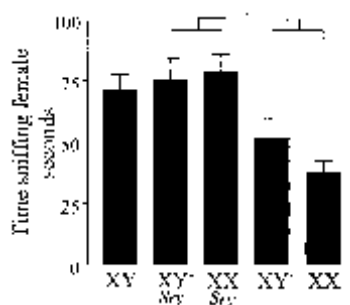
Males displayed more total mounts than females ( $F_{(1,67)} = 12.13$ ;  $p < 0.001$ ) (figure 53.2), but there was no significant effect of sex chromosomes or a significant interaction. None of the other behavioral measures (frequencies of mounts with thrusts or numbers of mounts in the first 30 min after mating) common to both males and females were significantly different. Total numbers of mounts tended to be greater for XY<sup>-</sup>Sry males than for XY males ( $F_{(1,23)} = 4.07$ ;  $p = 0.057$ ) (figure 53.2), but none of the other variables showed any statistically significant differences. There was a trend for the interval between the onset of intromissions and ejaculation to be shorter in XY males ( $32.2 \pm 8.4$  min) than in XY<sup>-</sup>Sry males ( $66.6 \pm 6.95$  min;  $F_{(1,17)} = 4.28$ ;  $p = 0.056$ ).

To determine whether animals of different genotypes were more or less likely to engage in certain types of



**Figure 53.2**

Total mounts (means  $\pm$  SEM) during sexual behavior tests with receptive female partners. Within the core cross, males (XY<sup>-</sup>Sry and XXSry) mounted more than did females (XY<sup>-</sup> and XX) ( $p < 0.001$ ). Groups sizes for groups left to right were 8, 15, 16, 15, and 13.

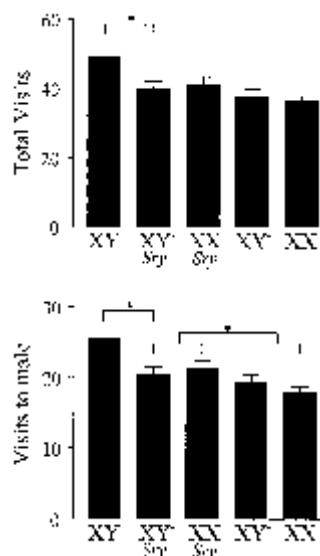


**Figure 53.3**

Time spent sniffing an anesthetized female in a 10 min social exploration test (means  $\pm$  SEM). Within the core cross, males (XY<sup>-</sup>Sry and XXSry) spent more time sniffing the stimulus female than did females (XY<sup>-</sup> and XX) ( $p < 0.0005$ ).

masculine behavior, we conducted  $\chi^2$  and Fisher exact tests. When a  $2 \times 2$  analysis was done to examine the effect of sex chromosomes or an interaction of sex chromosomes and sex, we found that the numbers of mounters and nonmounters did not differ among the four groups. However, when we examined the frequency of animals that did and did not display mounts with thrusts, we found that males were more likely to thrust than females ( $\chi^2(3) = 13.3$ ;  $p < 0.01$ ). No differences were found in the frequencies of XY and XY<sup>-</sup>Sry males that mounted, mounted with thrusts, mounted with intromissions, or ejaculated.

**Social Exploration** Males spent significantly more total time sniffing, particularly sniffing stimulus females, than did females (main effect of sex,  $F_{(1,62)} = 13.60$ , 18.37 for all sniffing and sniffing a female, respectively;  $p < 0.0005$ ) (figure 53.3), but there were no significant effects of sex chromosomes nor was there an interaction. There was a trend for a sex difference in the total number of visits [males visited more than females ( $F_{(1,68)} = 3.94$ ;  $p = 0.051$ )] (figure 53.4), and males vis-



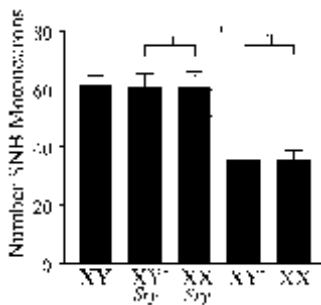
**Figure 53.4**

Numbers of visits (means  $\pm$  SEM) to the chambers in the three-chambered box, regardless of whether it contained a male or female stimulus animal (top), and visits to the chamber that contained the anesthetized male in the social exploration tests. Males (XY<sup>-</sup>Sry and XXSry) paid more visits to the stimulus male than did females (XY<sup>-</sup> and XX) ( $p < 0.05$ ). XY males paid more visits to the chambers with the stimulus male or female and also visited the stimulus male more often than did XY<sup>-</sup>Sry males ( $p < 0.05$ ).

ited stimulus males more often than did females (main effect of sex,  $F_{(1,68)} = 4.27$ ;  $p < 0.05$ ) (figure 53.4). XY males did more visiting and visited the anesthetized males more frequently than did the XY<sup>-</sup>Sry males ( $F_{(1,25)} = 5.37, 4.68$ , respectively;  $p < 0.042$ ) (figure 53.4). When the male groups were compared using a nested ANOVA, the difference was also significant for visits to the male ( $F_{(1,25)} = 5.32$ ;  $p < 0.044$ ). A trend in this same direction was noted for visits to the female ( $F_{(1,25)} = 3.62$ ;  $p = 0.07$ ; data not shown).

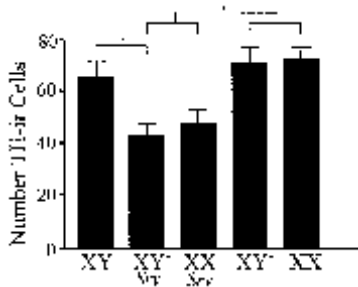
**Spinal Nucleus of the Bulbocavernosus** The number of SNB motoneurons was greater in males than in females (figure 53.5), replicating the sex difference found in rats and mice (Breedlove and Arnold, 1980; Wee and Clemens, 1987; Hauser and Toran-Allerand, 1989; Wagner and Clemens, 1989). In the two-way ANOVA, there was a significant effect of sex ( $F_{(1,31)} = 33.9$ ;  $p < 0.0001$ ), but no significant effect of sex chromosomes and no significant interaction. The male groups did not differ significantly from each other.

**TH-ir Neurons in the AVPV** Among the four genotypes that were progeny of XY<sup>-</sup>Sry fathers, females (XX and XY<sup>-</sup>) had more TH neurons in the AVPV than did their brothers (XY<sup>-</sup>Sry and XXSry) ( $F_{(1,50)} = 27.8$ ;  $p = 0.000003$ ) (figure 53.6). There was no significant effect of sex chromosomes and no significant inter-



**Figure 53.5**

Number of SNB motoneurons (means  $\pm$  SEM). Males of the core cross (XY<sup>-</sup>Sry and XXSry) had more SNB neurons than females (XY<sup>-</sup> and XX) did ( $p < 0.0001$ ) but did not differ from XY males derived from a different cross.



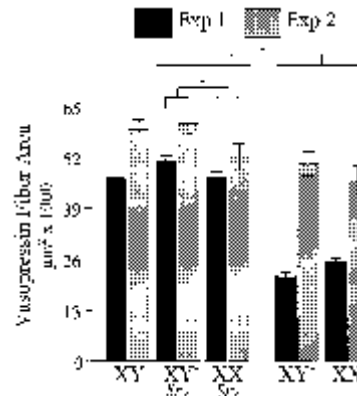
**Figure 53.6**

Number of TH-ir neurons in the AVPV (means  $\pm$  SEM). Within the core cross, males (XY<sup>-</sup>Sry and XXSry) had fewer neurons than females (XY<sup>-</sup> and XX) did ( $p < 0.000003$ ). Paradoxically, XY males had a feminine number of TH-ir neurons and therefore significantly more TH-ir neurons than did XY<sup>-</sup>Sry males ( $p = 0.08$ ).

action. XY males were significantly different from XY<sup>-</sup>Sry males in both the basic and nested ANOVAs ( $F_{(1,19)} = 9.23$  and  $13.2$ ;  $p < 0.008$ ). Related to this is the result that XY males did not differ from XX in the number of TH-ir neurons, in distinct contrast to C57BL/6 mice, which show a difference between XX and XY (Simerly et al., 1997).

### Experiments 1–3

**Vasopressin Fiber Density in the Lateral Septum** In Experiment 1, the tissue for males and females was fixed and immunostained at different times, so that the most appropriate comparisons in that experiment are within sex (figure 53.7). In experiment 1, XY<sup>-</sup>Sry males were more masculine (greater fiber density) than XXSry males, but XY<sup>-</sup> females were less masculine than XX females. Males had higher density of VP-ir fibers than females, which replicates the sex difference found consistently in rats and mice (Mayes et al., 1988; De Vries and Al-Shamma, 1990; Wang et al., 1993). The magnitude of this sex difference found in experiment 1, however, could have been partly an artifact of staining

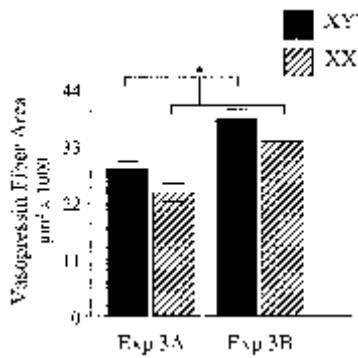


**Figure 53.7**

Density of vasopressin immunoreactive fibers in the lateral septum (means  $\pm$  SEM). Black and hatched bars represent the results from the first and second experiments, respectively. Within the core cross, males (XY<sup>-</sup>Sry and XXSry) had a higher density than did females (XY<sup>-</sup> and XX) ( $p < 0.000001$ ). XY<sup>-</sup>Sry males had a higher density than XXSry males ( $p < 0.05$ ), showing an effect of sex chromosome complement on this trait.

differences caused by separate processing of male and female tissues. In experiment 2, all tissue was stained and analyzed at the same time. The difference between XY<sup>-</sup>Sry and XXSry males was replicated in experiment 2, but not the difference between XY<sup>-</sup> and XX females. The three-way ANOVA showed a main effect of sex chromosomes ( $F_{(1,85)} = 4.9$ ;  $p = 0.0296$ ), and a main effect of sex [males greater than females ( $F_{(1,85)} = 90.6$ ;  $p < 0.000001$ )]. Planned comparisons indicated that there was an effect of sex chromosomes in males but not females (Tukey–Kramer;  $p < 0.05$ ). There was a significant interaction of experiment number and sex chromosomes ( $F_{(1,85)} = 4.63$ ;  $p < 0.034$ ) because the difference between XY<sup>-</sup> and XX, collapsing across sex, emerged in the second but not the first experiment (Tukey–Kramer;  $p < 0.05$ ). There was a significant interaction of experiment number and sex ( $F_{(1,85)} = 16.4$ ;  $p < 0.00012$ ), which reflects the artificially larger difference between sexes in the first experiment than in the second experiment. There was no main effect of experiment number. There was no significant difference between XY and XY<sup>-</sup>Sry males ( $F_{(1,37)} = 2.62$ ;  $p > 0.05$ ).

Because XY<sup>-</sup> females had higher density of VP-ir fibers than XX females in experiment 2 but not experiment 1, we examined this dependent variable in females derived from all-female litters in experiment 3. In this experiment, two batches of animals were stained and analyzed at different times and then compared in a single two-way ANOVA. XY<sup>-</sup> females had a significantly higher density of VP-ir fibers than XX females ( $F_{(1,43)} = 6.37$ ;  $p < 0.02$ ) (figure 53.8). There was no interaction between order of the experiment and genotype.



**Figure 53.8**

Density of vasopressin immunoreactive fibers in the lateral septum in mice from all-female litters (means  $\pm$  SEM). XY<sup>-</sup> females had a higher density than XX females did ( $p < 0.02$ ).

## Discussion

By measuring sexually dimorphic neural and behavioral traits in mice of one sex that possess different sex chromosomes (XY vs XX), we hoped to determine whether the sex chromosomes contribute to normal sexual differentiation of the brain and behavior. Our hypothesis was that a masculine complement of sex chromosomes (XY) causes the brain to develop in a more masculine and less feminine manner, compared with a feminine complement (XX). The panel of sexually dimorphic brain and behavioral phenotypes chosen for analysis included masculine copulatory and social behaviors known to be expressed more in males than females, and neural dimorphisms that are either male biased (SNB, septum) or female biased (AVPV) and occur at various levels of the neuraxis. Because development of these sexual dimorphisms is known in each case to be potentially influenced by gonadal secretions (Wagner and Clemens, 1989; Wang et al., 1993; Rissman et al., 1997; Wersinger and Rissman, 2000a), we suspected that any effects of sex chromosomes might be subtle.

We detected an effect of the sex chromosomes on one measure, the density of VP-ir fibers in the lateral septum. XY<sup>-</sup>Sry males were more masculine on this trait than XXSry males, and when females from all-female litters were examined, XY<sup>-</sup> females were more masculine than XX females. In contrast, there was no effect of sex chromosomes on the other phenotypes in the XX females, XY<sup>-</sup> females, XY<sup>-</sup>Sry males, and XXSry males, although these phenotypes varied by sex. The results suggest that sex chromosome genes contribute to some sex-specific patterns of brain differentiation. However, this contribution appears to be much smaller for the traits analyzed than the organizing effects of gonadal hormones, because the differences between male and female mice of the same

chromosomal sex were much larger than the differences between XY<sup>-</sup> and XX mice of the same sex. Because the relative variability in exposure to androgens from adjacent male fetuses is a plausible source of variability in the phenotype (vom Saal and Bronson, 1978), modest effects of intrauterine androgen may have masked the relatively smaller effects of sex chromosomes on VP-ir fiber density in females in the first experiment. The consistently higher VP-ir fiber density found in XY mice in the all-female litters in the third experiment supports this possibility. Because a masculine complement of sex chromosomes (XY<sup>-</sup>) led to greater masculinization of this trait than did a feminine complement (XX), the difference between XY<sup>-</sup> and XX mice is in the same direction as the sex difference in this trait. Although we have emphasized direct sex chromosome gene influences on neural development, the difference between groups in the present experiments could be the result of sex chromosome gene actions on any tissue that influences neural development.

The sex chromosome effect could originate via a number of different genetic mechanisms. (1) Y genes (encoded on the nonrecombining region of the Y) might masculinize the trait or inhibit feminine development. Sry and six other Y-linked genes are reported to be expressed in mouse brain (Kay et al., 1991; Agulnik et al., 1994; Zambrowicz et al., 1994; Lahr et al., 1995; Greenfield et al., 1996; Ehrmann et al., 1998; Mayer et al., 1998; Mazeyrat et al., 1998; Xu et al., 2002). The sex chromosome effect cannot be attributed to the action of Sry, however, because Sry was present as a transgene in the genome of both XY<sup>-</sup>Sry and XXSry males, and was absent in both female groups. (2) A double dose of one or more X genes might inhibit masculine development or promote feminine development of the trait in XX mice. Although most X genes are thought to be expressed in a single dose in each XX somatic cell because of inactivation of one of the X chromosomes, some X genes escape inactivation (Carrel et al., 1999) and could therefore be present in the brain in a higher dose in XX than in XY mice, as has been documented for several X genes (Xu et al., 2002). The X chromosome appears to have an unusually high number of genes implicated in brain-specific functions, which could contribute to the observed sex chromosome effect (Zechner et al., 2001). (3) Parent of origin effects (maternal or paternal imprinting) could activate or inactivate X chromosome genes differentially on maternally or paternally derived X chromosomes (Leighton et al., 1996; Skuse et al., 1997). Because XY<sup>-</sup> mice have only a maternally derived X chromosome, whereas XX mice have X chromosomes derived from both parents, these imprinting effects could give rise to different X gene dosages in XY<sup>-</sup> versus XX brain.

The present data support the idea that a masculine genome has a masculinizing effect on the density of septal VP-ir fibers and that this sex chromosome effect is not found in other sexually dimorphic CNS systems (SNB and AVPV) or in several measures of reproductive and social behaviors mediated by diverse brain circuits. Might XY<sup>-</sup> and XX mice have experienced different levels of gonadal or adrenal steroids, so that this effect of the sex chromosomes represents merely another example of the well known masculinizing effects of testosterone? Indeed, perinatal testosterone injections masculinize septal VP fiber density in rats (Wang et al., 1993). The sex chromosome effect reported here, however, is unlikely to be mediated by group differences in androgen secretion. Had the XY<sup>-</sup> genotype caused a greater secretion of testosterone perinatally relative to the XX genotype, the difference in these groups should have been detected in more than one phenotype because most of the phenotypes measured are masculinized by testosterone or its metabolites, probably during different but overlapping critical periods (Vale et al., 1973; Wagner and Clemens, 1989; Wang et al., 1993; Simerly et al., 1997). Thus, each system can be considered a sensitive barometer of levels of gonadal steroids during perinatal development, and the lack of sex chromosome effects in most of the systems is evidence against a sex chromosome effect on the levels of circulating gonadal steroids. We conclude that the sex chromosome effect is specific to only one of the hormone-sensitive phenotypes that we measured and does not reflect a broad increase in steroid secretion or action. More interesting would be a sex chromosome effect on the cellular and molecular systems that respond to gonadal steroids (e.g., receptors, receptor cofactors, etc.), or on the level of sex steroid synthesis in the brain itself (Schlinger et al., 2001). Whatever the molecular mechanism, the sex chromosome effect does not require a masculine endocrine environment because it was detected in both males and females.

Previous results could be interpreted to suggest that factors other than gonadal hormones contribute to sex differences in VP-ir fiber density. In rats, neonatal gonadectomy of male rats reduced the VP-ir fiber density to the level of control females, whereas neonatal testosterone treatment prevented these changes. Neonatal testosterone treatment of females, however, failed to increase VP-ir fiber innervation to that of control males (Wang et al., 1993). Although this discrepancy in testosterone effect is consistent with a sex chromosomal effect on the sexual differentiation of VP-ir fiber density, prenatal gonadal hormone levels may have contributed to sex differences in testosterone sensitivity. Because it is difficult to mimic throughout development a female endocrine environment in XY males or

a male endocrine environment in XX females, it has previously not been easy to eliminate sex differences in endocrine effects to focus on a role for the sex chromosomes in brain development. The genetic approach outlined in this paper therefore offers significant advantages for analyzing the relative contributions of sex chromosomal genes versus gonadal hormones in sexual differentiation.

The failure to find a sex chromosome effect on the number of TH-ir neurons in the AVPV in the present study might appear to conflict with the finding of Beyer et al. (1991) that female cultures of rat embryonic diencephalic neurons express higher levels of dopamine (see also Sibug et al., 1996). Because Beyer et al. (1991) attributed this sex difference to nonhormonal factors such as cell-autonomous actions of genes encoded on the sex chromosomes, one might have expected a sex chromosome effect on the number of TH-ir neurons in the AVPV. The lack of an effect could indicate, for example, that sex chromosome effects are exerted on diencephalic dopamine neurons in areas other than the AVPV, or that the effects found *in vitro* are not manifested *in vivo* (Reisert et al., 1990; Lieb et al., 1996). Interestingly, mesencephalic neurons from the same four sibling genotypes of the present experiment (XY<sup>-</sup>, XX, XY<sup>-</sup>*Sry*, XX*Sry*) were recently grown *in vitro* using conditions similar to that of Beyer et al. (1991), and a strong sex chromosome effect could be detected (Carruth et al., 2002). That is, cultures consisting of XY<sup>-</sup> or XY<sup>-</sup>*Sry* cells developed more TH-ir neurons than those derived from XX or XX*Sry* cells, so that sex chromosome complement, not gonadal status of the embryos, was the major determinant of group differences in TH neuron number. It is not yet known whether this sex chromosome effect is found in dopamine neuron populations *in vivo*.

XY males differed from XY<sup>-</sup>*Sry* by several measures: latency to thrust, the number of total visits and visits to males in the social exploration tests, and the number of TH-ir AVPV neurons. These differences are potentially attributable either to the fact that XY and XY<sup>-</sup>*Sry* come from different crosses (thus the groups may have experienced different environments or had uncontrolled differences in genetic background) or to a difference in the effect of the *Sry* transgene versus that of endogenous *Sry*. The latter seems more likely, given that the nested litter analysis showed that the variation among litters within groups, which is the result of environment and chance genetic variation, is exceeded by the variation between litters across groups, where the difference in *Sry* comes into play. These behavioral differences could reflect *Sry* effects on the brain or other tissues. The *Sry* transgene mRNA may be expressed at higher levels in embryonic gonadal ridge than that encoded by the endogenous *Sry* (A. Swain,

unpublished observations). Because the differences between XY and XY<sup>-</sup>*Sry* males were found in several but not all phenotypes, it is not clear whether the effect of *Sry* is mediated by an increase in androgen secretion or via a nonhormonal mechanism. Alternatively, the effects could be on nongonadal tissue outside of the brain. For example, the number of total visits and visits to males in the social exploration tests may be caused by differences in olfactory responsiveness (Paredes et al., 1998; Dominguez-Salazar et al., 2002). However, given that hypothalamic dopamine has been implicated in male sexual behavior (Hull et al., 1999), *Sry* effects on TH-ir neurons (Beyer et al., 1991) and male sexual behavior may be related.

Previous studies in mice have proved or suggested that the Y chromosome contains genes that influence various neural and behavioral traits, including aggressive behavior (Maxson et al., 1979; Maxson, 1992, 1999; Roubertoux et al., 1994; Guillot et al., 1995; Sluyter et al., 1996), the distribution of hippocampal mossy fibers (Hensbroek et al., 1995), dopamine systems (Sluyter et al., 1995), and brain serotonin (Tordjman et al., 1995). Moreover, Morris water maze learning performance has been reported to be more masculine in C57BL/6J XY<sup>POS</sup> female mice than in XX females (Stavnezer et al., 2000), although the fetal gonads of such females might contain some testicular tissue (Taketo et al., 1991), which could cause masculinization via a hormonal mechanism. These studies, together with the present findings, are consistent with a role for sex chromosome genes in neural and behavioral sexual differentiation. Interestingly, the same Y chromosomal factors that influence aggressive behavior (Sluyter et al., 1996) influence VP-ir fiber density in the lateral septum of mice selected for short or long attack latencies (Compaan et al., 1993) and may therefore have contributed to the differences found in the present study. These differences in VP-ir fiber density and aggressive behavior may also be causally related because VP has been implicated in aggressive behavior in rats and voles (Koolhaas et al., 1990, 1991; Winslow et al., 1993).

We have introduced a powerful model system for examining the separate and interactive effects of sex chromosomes and gonadal secretions on sexually dimorphic phenotypes. The dissociation of chromosomal and gonadal sex in these mice allows, for the first time, a strong test of the direct role of sex chromosomes in sexual differentiation of the brain and other somatic tissues. Importantly, these mice offer the ability to test the role of a masculine (XY) versus feminine (XX) complement of sex chromosomes under both masculine and feminine hormonal conditions. Group differences are sensitive to the effects of both X- and Y-linked genes. These mice will prove useful in further

studies to investigate the role of sex chromosomes in differentiation of other sexually dimorphic phenotypes and the molecular basis for sex chromosome-induced somatic sexual differentiation.

Although the present results indicate that at least one sexual dimorphism in mouse brain is influenced by the complement of sex chromosome genes, the results are also compatible with the strong web of evidence that has already proven the dominant role for gonadal steroids in the induction of sex differences in brain and behavior (Arnold, 2002; De Vries and Simerly, 2002).

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## Introduction

One of the most enduring controversies in the biological sciences is whether genetic constitution (i.e., "nature") or environmental influence (i.e., "nurture") has the uppermost hand in shaping the individual. To the proponents of eugenics of the latter part of the last century, nature and nurture were regarded as having parallel, non-interacting, influences on the organism, with nature being by far the most important ("omnicompetent") (1). Such a dualistic view had dangerous political and social implications, for it reinforced class divisions, discouraged upward social mobility through education, and encouraged ideas about limiting the gene pool through forced sterilization and other measures. One of the principal scientific reasons for rejecting the dualistic non-interacting model is that we now know that the genome of an organism is continuously called upon throughout the life cycle to make adjustments to the environment. Thus, the genetic constitution sets the limits by determining the type of gene product which will be expressed, but the environment plays a major role in selecting the amount and timing of gene expression. In other words, there is no longer a duality regarding environmental and genetic influences—rather, we now see that they work together and we are in the process of discovering the many ways in which this interaction takes place.

Nowhere is this shift in attitude more significant than in studies on the nervous system. Long regarded as a complex electrical control box or a biological computer, the brain is now recognized more and more for its plasticity to respond to and withstand environmental challenges, to change with use and experience, and to respond to internal hormonal signals. A turning point may have been the finding that the hereditary disease, phenylketonuria, did not necessarily produce mental retardation, providing the diet of the affected infant was carefully modified to reduce intake of phenylalanine (2). As another example of environmental impact, the studies of "enriched environment" (3) provided important insights as to how the brain of ge-

netically normal animals can be induced to grow and improve functionally as a result of environmental input even in old age (4, 5).

As a result of this change of emphasis, we now pay greater attention at the cellular level to the Nissl substance of neurons, i.e., the ribonucleic acid abundantly present in the perinuclear cytoplasm. One of the pioneers in studying this aspect, Holger Hydén, described the modifiability of neuronal and glial RNA content as a result of activity, as well as learning (6, 7). These studies pointed to the dynamic nature of genomic activity in the mature nervous system.

With the discovery around 1960 of intracellular receptor sites for steroid hormones (8), a new dimension was added to the study of genomic activity in the form of identified intracellular regulators of gene expression. We now know that steroid hormone receptors regulate gene expression by binding to enhancer-like regions of DNA, and that this interaction increases or decreases transcription of nearby genes (9). There is another aspect of hormone action which is crucial to understanding how the environment regulates gene expression in brain, namely, the role of the CNS as controller as well as target of hormones. Hormone secretion by the adrenal glands and gonads is under the control of the brain by way of hypothalamic releasing factors and pituitary trophic hormones. Neural control of the endocrine system involves diurnal and seasonal rhythms entrained by the light-dark cycle and by day length, as well as stimulation and inhibition of hormone secretion by experiences and by behavior. Steroid hormones thus offer unique opportunities for exploring environmental control of gene expression in the brain. They produce a wide range of effect beginning in early development, continuing into adult life and into senescence, and include both the repair and causation of brain damage. In this article, we examine the features of steroid hormone action on the brain which best illustrate the impact of "nurture" upon "nature." We shall first consider the activation of gene expression by steroids and then examine the range of effects of steroid hormones on brain function throughout the life cycle.

## Steroid Hormone Effects on Gene Expression

### Receptors

Steroid hormone receptors are proteins which contain binding domains for the steroid as well as for DNA, and the function of the steroid hormone is to catalyze a conformational change in the receptor which exposes the DNA-binding domain so that it can interact with enhancer-like elements in the genome (9). Although it is clear that the final cellular site of action of these receptors is the cell nucleus, there is some controversy as to where in the cell the receptor resides when the steroid is not present. Originally conceived to be cytoplasmic proteins which translocate to the cell nucleus when steroid is present (10), steroid receptors are known to reside in cell nuclei in some cells (11) and in cytoplasm of other cells (12) when there is no steroid available. It is not clear why such differences exist, but the differences do not appear to be important for the purposes of the present discussion.

### Genomic Involvement in Lordosis Behavior

Let us now consider an example of how steroid hormones induce changes in genomic activity in the nervous system, in order to better understand the relationship between gene expression and the impact of hormones on behavior and brain structure and function (13). In adult life, the female rat has a reproductive cycle of 4–5 days duration, during which estradiol (E) and progesterone (P) are secreted sequentially in response to an internal, light-dark entrained rhythm and feed back on the brain to induce sexual activity (lordosis behavior) which is temporally coordinated with ovulation to increase fertility. A key feature of studying lordosis is the ability to localize a discrete brain region. It is fortuitous that E and P actions can be localized to a small group of neurons within the ventrolateral part of the ventromedial nuclei of the hypothalamus (VMN), and the study of these neurons' response to hormone provide a unique glimpse into the reversible modulation of genomic activity by steroid hormones. One of the earliest responses to E treatment, occurring within 2 hours, is the hypertrophy of the VMN neurons, with increases in both cell body volume as well as cell nuclear and nucleolar volume. Increased amounts of stacked rough endoplasmic reticulum are accompanied by increased hybridizable 28S ribosomal RNA. Altered patterns of protein labelling is also found, which changes with time after E treatment, suggesting a cascade of induced gene expression. Within the time-frame in which these events are taking place, the application of protein and RNA synthesis inhibitors block the induction of lordosis behavior. Moreover, E-induces gene products such as progesterin receptors, which enable the VMN to respond to the se-

quential secretion of progesterone. Moreover E induces muscarinic cholinergic receptors in VMN, and this induction is also found to be temporally related to induction of lordosis. Besides acetylcholine, neurotransmitters such as serotonin, norepinephrine (NE), GABA, oxytocin and cholecystokinin (CCK) also play a role in controlling lordosis behavior in the vicinity of the VMN, and E and P may be involved in regulating the influences of many of these transmitters on VMN neurons (13).

### Steroid Hormones Do Not Work Alone

As straight-forward and satisfying as are the actions of E and P on VMN neurons, it must not be assumed that the steroid hormones work alone in producing their effects. In fact, other cellular regulators contribute to steroid regulated gene expression, and they do so in at least two ways. One of these is to modify the steroid receptors themselves. For example, in the VMN of the guinea pig, alpha adrenergic drugs alter the number of estrogen-inducible progesterin receptors (14) and at the same time influence the display of lordosis behavior (15). The mechanism is not known, but it could conceivably involve modifying the receptors through phosphorylation or dephosphorylation. Similar modulation of estrogen receptors in pituitary and hypothalamus has been recently reported for a variety of neurotransmitter-related drugs (for references, see 16).

Another way in which cellular regulators contribute to steroid-regulated gene expression is by exerting parallel or competing effects on gene expression. A recent example of this concerns the ability of estradiol to alter expression of the tyrosine hydroxylase (TH) gene in the arcuate nucleus of the hypothalamus (17). By itself, E increases TH gene expression as shown by a transcription run-on assay; prolactin by itself exerts a parallel stimulation of TH transcription. However, in the presence of anterior pituitary tissue, E causes a decrease in transcription. What appears to be happening is that E stimulates secretion of another hormone by pituitary which overrides, or at least changes, the nature of the E effect in the arcuate nucleus. The identity of the pituitary hormone is not known; conceivably, it might be prolactin itself, for E is known to stimulate prolactin transcription and secretion. The existence of this and other types of regulatory interactions between hormones must be borne in mind in unravelling hormone actions on neuroendocrine function and behavior.

## Steroid Hormone Actions Throughout the Life Cycle

### Background

The second major feature of steroid hormones as agents linking "nature" and "nurture" is the wide range of effects which they exert on the nervous sys-

tem, beginning during embryonic life and extending throughout adult life and into senescence. The purpose of this section of the article is to review some of these effects and their overall physiological significance.

### Brain Sexual Differentiation

During perinatal development in mammals, the embryonic testicular hormone, testosterone (T), initiates cellular events which determine that the brain will develop in a masculine direction irrespective of the genetic sex: i.e., in this case, "nurture" supercedes "nature" (18). That is, female rats given T as neonates will not develop masculine traits and will, instead, display sexual responses to hormone priming in adulthood which are similar to those of males, and males castrated as neonates will display feminine characteristics as adults.

Both androgens and estrogens are involved in brain sexual differentiation through the conversion of T to estrogens and to more potent androgens. The actions of T on the brain to promote masculine sexual differentiation involve the developmental expression of receptors for androgens and estrogens and enzymes which convert T to estradiol (aromatizing enzymes) and to 5 alpha dihydrotestosterone (5 alpha reductase). Androgen and estrogen receptors are expressed beginning prenatally and are expressed equally in both sexes. In the case of estrogen receptors, we know that expression becomes detectable several days after the final cell divisions of specific groups of hypothalamic and preoptic area neurons, and they are expressed in equal amounts in both males and females (18). Thus the receptors are present when perinatal T secretion occurs in the male, and they are occupied by E derived via local aromatization from circulating T (18). A similar pattern of selective occupancy in the male of receptors found equally in male and female brains occurs for androgen receptors (18).

How does T produce these effects on the developing brain? One early response to estrogen derived by aromatization from T is an increased amount of neurite outgrowth and branching, which may lead to increased terminal fields and cell body size (19). Another aspect of T action is to promote differentiation of the ability of the adult CNS to respond to hormonal signals in adult life. For example, male and female rat brains differ in response to E with regard to regulating serotonin receptors (20) even though E receptors are present in similar amounts in male and female brains (18). Moreover, male rats, as a result of early actions of T on the brain, are not responsive to progesterone (P) with respect to inducing feminine sexual (lordosis) behavior. One way in which this sex difference is seen biochemically is that E induces fewer P receptors in VMN of males than in females (21). This sex differences can be

reversed by neonatal manipulations of brain sexual differentiation which also reverse the phenotypic display of lordosis behavior in response to E and P (22).

### Activational Effects of Hormones on Brain Function

After the critical period of sexual differentiation, actions of gonadal hormones change from those affecting permanent features of brain structure and function to reversible actions that coordinate adult brain function. Those reversible effects which occur cyclically during natural rhythms of hormones secretion are called "activational" effects. One of the best examples is the cyclic activation of lordosis behavior in the female rat which is described above.

Other examples of "activation" derive from the actions of adrenal steroids on brain function during the course of the diurnal cycle of pituitary-adrenal activity (23). Adrenal steroid levels increase prior to the onset of daily activity and are, at least in part, responsible for elevated exploratory activity and food intake (23). In addition, adrenal secretions are involved in coordinating the time of peak synaptic efficacy so that it occurs during waking hours (23). Moreover, adrenal steroids delay sleep (24), thus further enhancing their importance for coordinating optimal performance during waking hours.

### Adaptation and Adrenal Steroids

Besides being secreted in a diurnal cycle which is subject to coordination by day length and by availability of food (23), adrenal steroid secretion is also responsive to experiential input in the form of increased work load, excessive heat or cold exposure, trauma, fear and frustration. This form of adrenal steroid secretion is referred to as "stress-induced," and the response of the brain to adrenal steroid secreted in "stress" is consistent with the concept of adaptation.

How does the brain keep track of adrenal steroid secretion in relation to the diurnal rhythm and to stress? It appears to do so by means of two different receptors each of which recognizes a different range of circulating corticosterone. Eighteen years ago, we reported that the hippocampus retains high concentrations of the glucocorticoid, [<sup>3</sup>H]-corticosterone, and our attention has focused upon this structure (25). One reason for this is the importance of the hippocampus for both cognition and affect. Moreover, glucocorticoids have been shown to alter both mood and cognitive performance. Thus far, our studies have focused primarily on the glucocorticoid receptors themselves, and they have suggested an interesting hypothesis of glucocorticoid action. Rather than one glucocorticoid receptor, there are two subtypes which were recently identified by Reul and DeKloet (26). One type, called Type I, has a higher affinity for corticosterone and is able to function

as a receptor in response to diurnal variation of corticosterone, which has a lower amplitude than the stress response. The Type I receptor is concentrated in hippocampus and appears to account for the uptake of [ $^3\text{H}$ ]-corticosterone referred to previously. We postulate that this receptor mediates biochemical responses which cycle as a result of the diurnal glucocorticoid rhythm (23).

The other glucocorticoid receptor, called a Type II receptor, which has a widespread distribution in the brain in both neurons and in glial cells, has a lower affinity for corticosterone and responds to stress levels of the hormone as well as to synthetic steroids such as dexamethasone. We believe that this is the receptor which mediates effects of repeated stress on the brain, including effects in the hippocampus. As one example of such effects, various investigators have shown that repeated stress induces a decrease in the ability of NE to stimulate cyclic AMP (cAMP) accumulation in cortex, and that this effect is mediated by glucocorticoids (27). Glucocorticoids such as dexamethasone (DEX) also induce a decrease in NE- and VIP-stimulated cAMP accumulation in hippocampus (27). In addition to beginning to investigate the mechanism of the hormone-induced decrease, we have also been stimulated to ask whether there are sex differences in response to glucocorticoids in hippocampus. One reason for asking this question is that male and female rats differ in the degree to which they will adapt to repeated environmental stress—males adapt in terms of reduced behavioral depression more rapidly and completely than females (28). If desensitization of the NE response is part of the adaptive process, then one would predict that the female might not show this response as readily as males. Indeed, this appears to be the case, since DEX treatment fails to decrease the cAMP response to both NE and to VIP in females whereas it does so in males (29).

Is this sex difference in response to glucocorticoids in any way due to a sex difference in glucocorticoid receptors in hippocampus? The recent work of Turner and Weaver (30) indicates that females actually have higher levels of total glucocorticoid receptors than males, and our own recent work confirms this and shows that Type II receptors are somewhat higher in females than in males 15 hours after adrenalectomy (ADX) when endogenous steroid levels have cleared (31). However there is up-regulation of receptors with increasing time after ADX, and much to our surprise this up-regulation is more pronounced in males after ADX than in females (31). Thus, males again appear to be somewhat more sensitive to glucocorticoids than females, in agreement with the cAMP results described above. In neither case do we know yet to what extent the sex difference is the product of perinatal sexual dif-

ferentiation or to what extent it may be the product of the presence of circulating gonadal hormones in adulthood. Future experiments will be directed towards answering these questions.

#### **Down-Regulation of Receptors and Damaging Effects of Glucocorticoids**

What happens when adrenal steroid secretion or stress is prolonged? One response of target tissues such as hippocampus is to show down-regulation of glucocorticoid receptors, as if the tissue is reducing its sensitivity to circulating hormone (32). In spite of this down-regulation, further prolongation of adrenal steroid exposure results in tissue damage and neuronal loss in the hippocampus (32). Such neuronal loss mimicks loss of neurons which occurs with age (32).

One of the apparent consequences of such neuronal loss is the excess secretion of adrenal steroids which accompanies aging and is also associated with other instances where glucocorticoid receptors are down-regulated in hippocampus (32). This hypersecretion of glucocorticoids, along with ACTH and beta endorphin, is especially apparent in the aftermath of stress and appears to represent an attenuation of shut-off of the stress response. It has the potential to cause widespread damage throughout the body, ranging from immunosuppression to muscle atrophy, calcium loss from bone, steroid diabetes and increase growth of virally-induced tumors (32–34).

#### **Paradoxical Effects of Steroids—Regenerative Responses**

The story of steroid hormones as messengers of environmental influences on the nervous system would be incomplete without noting the fact that gonadal steroids and glucocorticoids can under different circumstances not only cause neural damage but also promote regenerative responses to other types of damage. Whereas chronic elevation of estrogens leads to the “hypothalamic disconnection syndrome” and disruption of estrous cyclicity in female rats (35), estrogens also promote synaptic sprouting and reinnervation within the hypothalamus after deafferentation (36), and androgens promote regrowth from the severed hypoglossal nerve (37). Finally, glucocorticoids promote homotypical sprouting of damaged serotonin fibers in hippocampus (38), even though they also inhibit collateral growth of other lesioned neural pathways (39).

#### **Conclusions**

We have seen that steroid hormones of the gonads and adrenals are messengers sent by signals originating in the brain and that they coordinate and regulate events in many organs of the body, including the brain, by acting upon the genome. We have also seen that

the brain is a target organ for hormone action on gene expression throughout the life cycle and that the nature of the hormone effects changes as the brain matures and ages. In particular, the actions of hormones during early development permanently modify neuronal growth and differentiation, whereas hormone actions on mature neurons reversibly modulate gene expression.

However, we have also seen that these same hormones can damage and even destroy neural tissue under conditions of prolonged exposure. Where are we to place these actions in relation to these other, largely beneficial, hormone actions? One answer to that cumulative damage brought about by hormone exposure may be an inevitable and natural part of the aging process and an unavoidable by-product of the other effects which these hormones have on brain function. Moreover, it is conceivable that individual differences in hormone secretion throughout a lifetime may influence individual patterns of aging. Another important consideration regarding damaging effects of hormones, which pertains to adrenal steroid secretion and stress, is that such damage is far from the most immediate consequence of stress. Rather, the overwhelming feature of glucocorticoid action in the aftermath of stress is its ability to assist in restoration of homeostasis. In fact, glucocorticoids counteract many of the body's defense mechanism that are activated by stress and prevent them from overreacting and damaging the organism (40). In the brain, this can be seen in several ways: Glucocorticoids shut off the neuroendocrine stress response; they also act upon the brain to counteract stress-elevated reactivity of noradrenergic and serotonergic neurons that is associated with behavioral depression and in this regard may act as a natural "anti-depressant" (23). Thus the most important feature of neurally-activated secretion of adrenal glucocorticoids is their ability to promote recovery and adaptation, rather than to cause damage. However, hormone exposure can also cause damage, and future studies must determine "how much is too much" and ascertain the chemical factors, besides the hormone itself, which determine the eventual transition from adaptive effects of glucocorticoids to degenerative actions.

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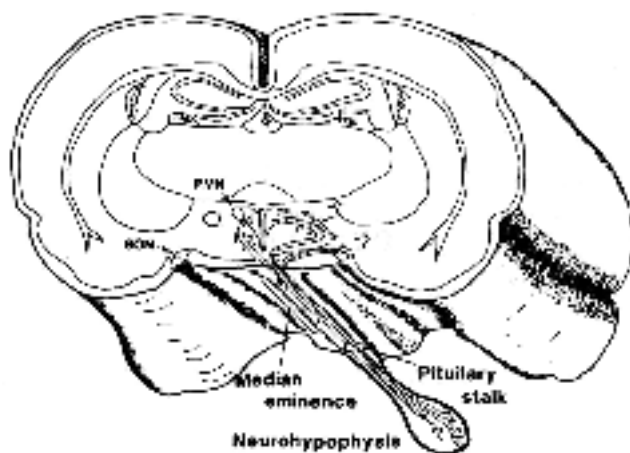
This chapter is concerned with rather remarkable recent discoveries demonstrating that the brains of maternally behaving rats are structurally different from those of other animals. The differences discussed here have been observed in the magnocellular hypothalamo-neurohypophysial system, but similar brain alterations may occur in other areas as well. Neurons in this system manufacture and release the peptide hormones oxytocin and vasopressin. Oxytocin is of particular interest because it is involved in producing the uterine contractions preceding and during parturition, as well as in producing the milk-ejection reflex in response to the suckling stimuli of the young. A new conceptualization of brain reorganization is supported by the findings reviewed here. The brains of maternally behaving animals can no longer be thought of as similar to those of naive virgin animals that simply have some pathways differentially activated by the circumstances of motherhood. Rather, the data support the idea of extensively reorganized cell-cell interactions. For example, new specialized synapses form on the dendrites of magnocellular neurons during or immediately after parturition. The neuronal cell bodies similarly receive new synaptic inputs, but in contrast to the dendrites, the cell bodies receive these novel synapses during lactation. Electrical coupling among these neurons appears to be inducible by olfactory and/or vomeronasal organ stimulation that directly activates oxytocinergic neurons. It is hypothesized that the anogenital licking of the pups in the period immediately prior to suckling is a powerful and crucially important stimulus to these two olfactory systems. It is felt that the consequent electrical coupling “fine-tunes” the neuronal interactions, maximizing the efficiency of the milk-ejection reflex, which occurs in response to suckling stimuli. Among the more intriguing recent findings in this system is the fact that virgin rats that have been induced by the continuous presence of young rat pups to show maternal behavior also display some of the same brain modifications found in the lactating, real mothers. Finally, nearly all the changes that have so far been found to occur in the brains of maternal rats disappear about a month after the pups are weaned.

### Anatomy of the Hypothalamic Magnocellular System

Among the many physiological events that accompany pregnancy, birth, and subsequent lactation in mammals is a significant increase in the synthesis and release of the peptide oxytocin. Along with vasopressin, oxytocin is synthesized in magnocellular neuroendocrine cells (MNCs) located in the hypothalamus, the two hormones being manufactured by separate cell populations. Although several accessory nuclei that manufacture these peptides have been identified (Peterson, 1966), the predominant nuclei of this system are the supraoptic nuclei (SON) and the magnocellular division of the paraventricular nucleus. These nuclei, their relative locations in the brain, and some of their axonal projections are diagrammed in figure 55.1. Several anatomical features distinguish MNCs from surrounding areas of the hypothalamus. As shown at the light microscopic level in figure 55.2, the nuclei are conspicuous because of their large, densely packed, densely staining somata (15–30  $\mu\text{m}$  in diameter) and their locations lateral to the third ventricle and at the ventral surface of the brain just lateral to the optic chiasm and optic tracts. Simply branching dendrites of paraventricular MNCs project medially toward the third ventricle (Armstrong, Warach, Hatton, & McNeill, 1980; van den Pol, 1982), while those of the SON initially project ventrally and turn to course in a parallel fashion rostrocaudally along the ventral surface of the brain (Armstrong, Schöler, & McNeill, 1982). In this chapter, the discussion will be confined largely to the SON (figure 55.3).

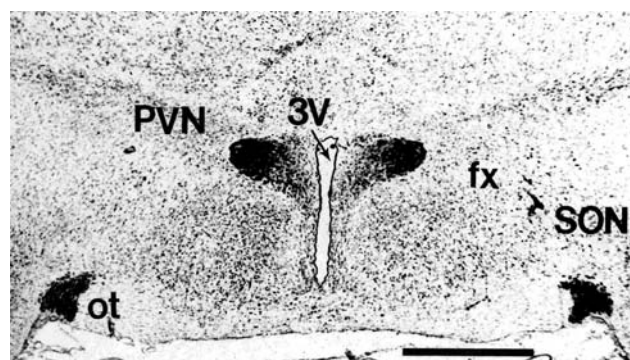
### Morphological Characteristics of Magnocellular Neurons

Electron microscopic observations have demonstrated that even though somata are densely packed, under basal conditions neighboring cells are isolated from one another by thin astrocytic processes (Hatton & Tweedle, 1982; Tweedle & Hatton, 1976, 1977) (figure 55.4A). Studies of the ventral dendritic zone of the SON have shown that thin astrocytic processes separate dendrites in a manner similar to that seen in the somatic region (Perlmutter, Tweedle, & Hatton, 1984,



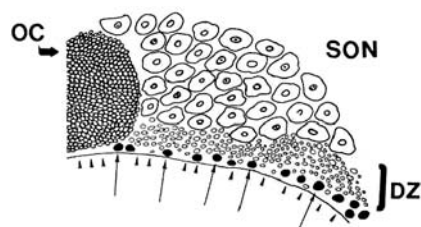
**Figure 55.1**

A three-dimensional drawing illustrating the location of the hypothalamo-neurohypophyseal system within the brain. Many cells in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) have axons that give off local collaterals in areas adjacent to the nuclei (open arrows). Parent axons continue and course through the median eminence and pituitary stalk to terminate in the neurohypophysis. Some axons from portions of the PVN also project to the median eminence.



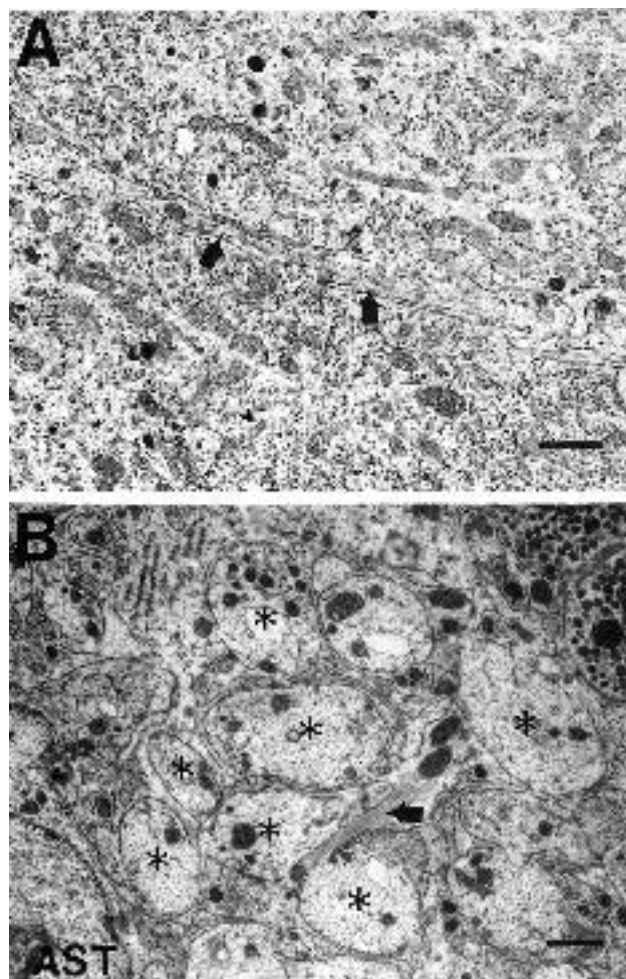
**Figure 55.2**

Thionin-stained coronal section of the hypothalamus. Magnocellular nuclei are easily identified by their densely stained and packed cell bodies. Abbreviations: PVN, paraventricular nucleus; SON, supraoptic nucleus; ot, optic tract; fx, fornix; 3V, third ventricle. Bar = 1 mm.



**Figure 55.3**

Schematic diagram of the supraoptic nucleus (SON), which is located immediately lateral to the optic chiasm (OC) and dorsal to the pial surface (marked by arrowheads). Dendrites from these cells form a distinct dendritic zone (DZ) ventral to the cell bodies. Astrocytic cell bodies (some of which are indicated by arrows) line the most ventral portion of this area.



**Figure 55.4**

Ultrastructural appearance of adjacent SON magnocellular neurons and dendrites in normal animals. (A) Tightly packed magnocellular cell bodies are isolated from one another by astrocytic processes (arrows). Bar = 1  $\mu$ m. (B) Individual dendrites (asterisks) in the dendritic zone are also separated by astrocytic processes. Astrocytic filaments (arrow) are commonly seen throughout the nucleus. Part of an astrocytic cell body (AST) is also seen. Bar = 1  $\mu$ m.

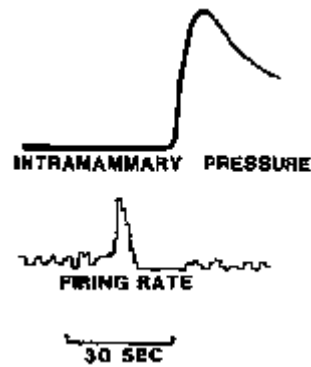
1985) (figure 55.4B). These dendrites, again under basal conditions, also possess an ultrastructural feature that is uncharacteristic of most neural tissue: they often have vacant or incompletely covered postsynaptic densities (Tweedle & Hatton, 1986). The cytoplasm of MNCs is richly endowed with numerous organelles indicative of their peptidergic nature. Most notable are many dense core vesicles that contain the secretory products of these cells. A well-developed Golgi apparatus, abundant free ribosomes, and diffuse endoplasmic reticulum allow these cells to be readily identified.

#### Magnocellular Projections to the Neural Lobe

MNCs have various projections within the brain stem and spinal cord; the functions and extent of these

connections are only now being investigated (Swanson, 1986). Some axons also give off collaterals that terminate in the hypothalamus in areas adjacent to the nuclear borders of both the paraventricular nuclei (Hatton, Cobbett, & Salm, 1985) and the SON (Mason, Ho, & Hatton, 1984). MNCs are best known for their massive projection to the neurohypophysis, which forms the hypothalamo-neurohypophysial tract. This tract is formed by axons from magnocellular neurons that course through the internal zones of the median eminence and terminate in the neurohypophysis (see figure 55.1). Oxytocin and vasopressin are released by terminals that contact the basal lamina surrounding fenestrated capillaries in the neural lobe of the pituitary. Action potentials that invade terminals cause the calcium-dependent exocytotic release of hormones into the circulation. Several factors may modify hormone release at the level of the neural lobe. A variety of neurotransmitters have been found in the neural lobe, and they may modulate the secretion of these hormones. Pituicytes, the astrocytes of the neurohypophysis (Salm, Hatton, & Nilaver, 1982; Suess & Pliška, 1981), may also modulate release through their association with the basal lamina and axonal terminals. Transmission and scanning electron microscopy have shown that under basal conditions, axonal terminals are often completely enclosed by pituicyte cytoplasm (Hatton, Perlmutter, Salm, & Tweedle, 1984; Tweedle & Hatton, 1980b, 1982). Thin pituicyte processes are also interposed between axonal endings and the basal lamina, through which hormone must pass in order to enter the capillary circulation (Tweedle & Hatton, 1987; Wittkowski, 1986). Evidence that these arrangements between axons, pituicytes, and the vascular system participate in modulating hormone release is presented later in this chapter.

Although the main efferent pathways of axons from the SON to the neurohypophysis are well established, less is known about afferent input to these cells. Intrahypothalamic connections, as well as input from various portions of the brain stem and limbic system, are extensive and complex. Catecholaminergic inputs reach the SON from brain stem nuclei, and many afferent inputs probably influence MNCs via local interneurons located in perinuclear areas. For example, a cholinergic input to the SON from local neurons lying just dorsal and lateral to the SON has recently been described (Hatton, Ho, & Mason, 1983; Mason, Ho, Eckenstein, & Hatton, 1983). Other probable inputs to the SON include  $\gamma$ -aminobutyric acid (GABA) from local neurons, angiotensin from the subfornical organ, serotonin and histamine from more posterior regions of the hypothalamus, and excitatory amino acid inputs from the olfactory system.



**Figure 55.5**

Tracings showing the temporal relationship between the firing rate in a magnocellular neuron and the milk-ejection reflex. Upper trace: pressure transducer. Lower trace: rate meter. A significant increase in firing rate precedes the rise in intramammary pressure, indicating a milk ejection. (Modified from Belin et al., 1984)

### Physiological Functions of Magnocellular Neurons

A wide variety of central effects for oxytocin and vasopressin have been proposed, including roles in memory consolidation and cardiovascular functioning (reviewed in Meisenberg & Simmons, 1983). These peptides are best known for their peripheral effects: oxytocin, for its role in the contraction of uterine smooth muscle during parturition and the milk-ejection reflex during lactation; and vasopressin, or antidiuretic hormone, as a promoter of water resorption by the kidneys. Typically, experimental manipulations that seek to understand the neurobiology of this system use either dehydrated animals, which causes both peptides to be released, or nursing animals in which there is a rather selective release of oxytocin. It should be recognized, of course, that vasopressin is also of great importance to the lactating animal in maintaining water balance during suckling.

### Electrophysiological Studies of Magnocellular Neurons

Electrophysiological studies have determined that oxytocinergic and vasopressinergic cells can be distinguished on the basis of their firing characteristics (see Poulain & Wakerley, 1982, for review). Presumed oxytocinergic cells have been characterized in anesthetized lactating rats with suckling pups. In these animals, MNCs may fire in either a "fast continuous" or a "slow irregular" manner; however, presumed oxytocinergic cells occasionally exhibit a synchronized, high-frequency discharge, after which milk ejection occurs. The relationship between these two events is shown in figure 55.5. Vasopressinergic cells do not generally respond to suckling, and, in osmotically stimulated rats, they display a phasic firing pattern consisting of alternating periods of silence and bursts of action

potentials (Cobbett, Smithson, & Hatton, 1986). A variety of mechanisms appear to participate in generating these stereotypic patterns of activity and in producing synchronous firing among oxytocinergic neurons. It may be that some of the many dramatic anatomical changes that occur in this system during activation contribute to the characteristic activity patterns observed. Although our emphasis here is on the alterations that occur in the hypothalamo-neurohypophyseal system of female rats during parturition and lactation, many of these changes can also be shown to occur in dehydrated animals of either sex (Perlmutter et al., 1985; Tweedle & Hatton, 1976, 1977, 1980a, 1982, 1984, 1986, 1987).

#### **Oxytocin's Role in Parturition and Lactation**

Ever since Dale (1906) discovered that pituitary extracts produce uterine contractions in pregnant cats, experimental work has increased our appreciation for and our knowledge of the role of oxytocin in animal parturition (for review, see Fuchs, 1985). A general conclusion from these experiments is that the hypothalamoneurohypophyseal system is activated prior to parturition (i.e., expulsion of the first fetus), with increasing activity measured as parturition is completed. Oxytocinergic cells increase their firing rates during parturition, and bursts of action potentials precede the expulsion of each fetus (Summerlee, 1981). Similarly, the pituitary content of both oxytocin and vasopressin is progressively depleted during parturition (Fuchs & Saito, 1971), a process reflected in progressive increases in blood levels of oxytocin (Higuchi, Tadokoro, Honda, & Negoro, 1986).

Oxytocin also plays a pivotal role during lactation, when it serves as the efferent limb of the milk-ejection reflex. Although the complete anatomical pathway for the milk-ejection reflex has not been worked out, it is well established that suckling of pups is synaptically relayed to excite oxytocinergic cells. Despite continuous suckling of pups, milk ejection does not occur continuously, but about every 10–12 min. Calculations of the number of oxytocinergic cells that must be activated in order to release enough hormone for milk ejections to occur suggest that most oxytocinergic cells in the magnocellular nuclei must fire within a very brief time span.

Electrophysiological studies have confirmed that this is indeed the case: just prior to milk ejections, oxytocin neurons fire bursts of action potentials (see figure 55.5). These bursts occur relatively synchronously both within and between the two nuclei (Belin & Moos, 1986; Belin, Moos, & Richard, 1984). Within seconds after this bursting, a milk ejection occurs. The milk-ejection reflex poses a number of continuing questions for neurobiologists. In addition to the paucity of information

about the precise anatomical pathway from the mammary gland to the oxytocinergic cells in the hypothalamus, and the lack of specific information on where and how stimulation from the mammary gland is gated, the mechanism by which oxytocinergic cells can synchronize their firing with such precision remains to be elucidated.

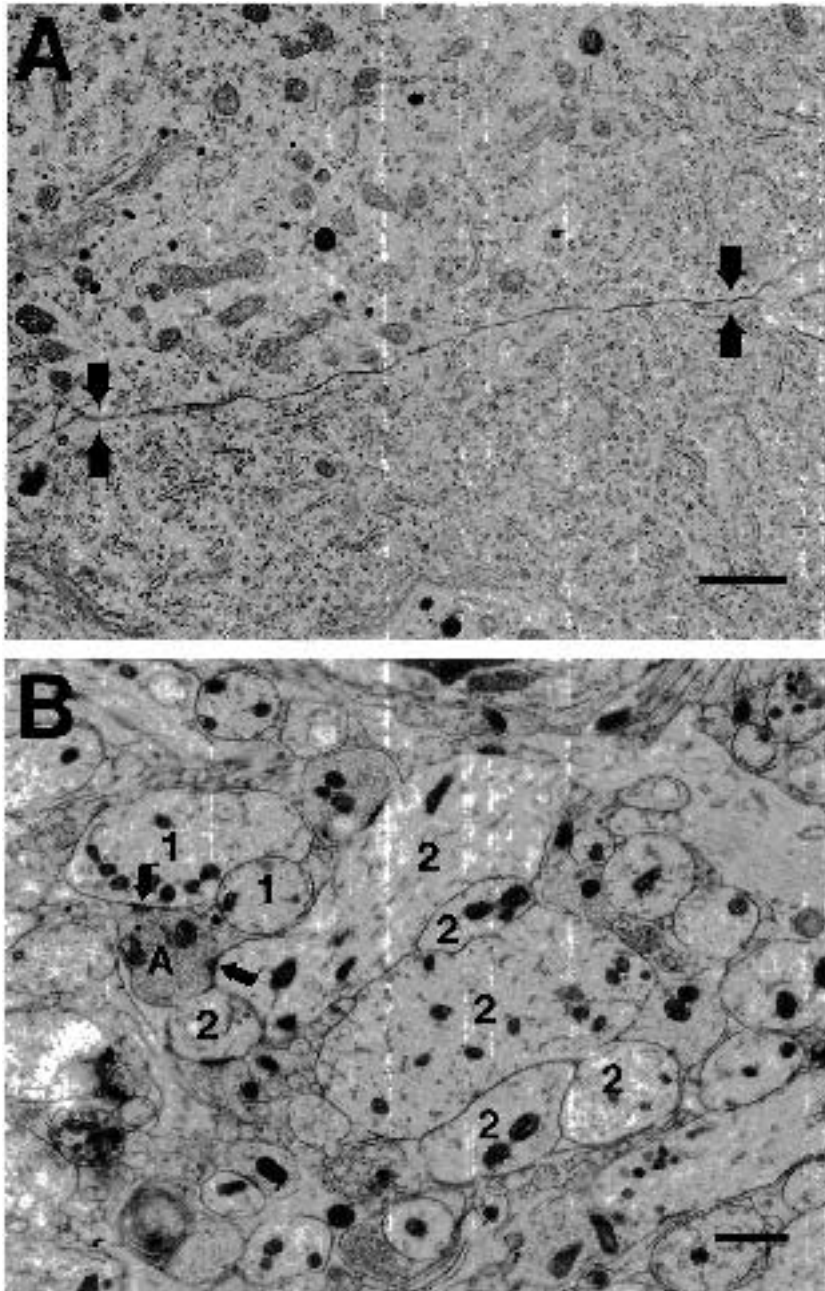
#### **Anatomical Transformations in Brain and Pituitary Associated with Parturition and Lactation**

Studies using both parturient and lactating females have provided experimental insights into the hypothalamo-neurohypophyseal system under physiological conditions of both acute and chronic demand. Given the heightened state of neuronal activity and hormone release during parturition and lactation, it is perhaps not surprising that the organization of the various neural elements of the hypothalamo-neurohypophyseal system in parturient and lactating females is dramatically different from that of virgin animals.

#### **Ultrastructural Changes in Glial–Neuronal Relationships**

Ultrastructural studies have revealed that a unique transformation in the cellular elements occurs in the SON around the time of parturition and during lactation. These changes are unusual in that they involve both the neurons and the astrocytes of the SON (Hatton & Tweedle, 1982; Theodosios, Poulain, & Vincent, 1981). In prepartum animals (2–24 hr prior to parturition), the glial processes that normally separate adjacent neurons and dendrites of the SON have withdrawn, resulting in a significantly higher proportion of direct neural membrane apposition (Hatton & Tweedle, 1982; Theodosios & Poulain, 1984) (figure 55.6A). These somatic appositions are maximally elevated after 14 days of lactation, when cellular membrane apposition is increased more than 30-fold compared with that of virgin animals (Hatton & Tweedle, 1982). That direct membrane appositions in lactating animals involve withdrawal of SON glial cell processes has been demonstrated in an immunocytochemical study using an antibody to the astrocytic marker glial fibrillary acidic protein. A redistribution of this marker is seen in the SON of lactating animals compared with virgin SONs (Salm, Smithson, & Hatton, 1985). This redistribution is not seen in control areas in the lateral hypothalamus, indicating that the effect is not a generalized hypothalamic response.

In the dendritic zone of the SON, dendritic bundles (i.e., two or more dendrites with a membrane in direct apposition) (figure 55.6B) form as a result of the withdrawal of glial processes (Perlmutter et al., 1984). Both the extent of dendritic membrane in apposition and the



**Figure 55.6**

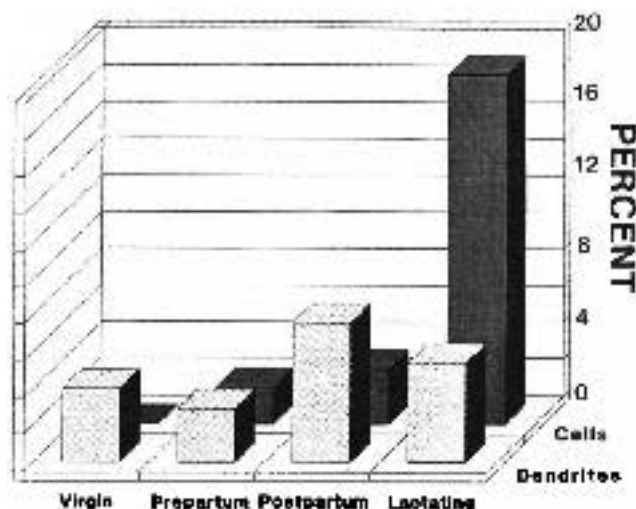
Ultrastructural appearance of adjacent SON magnocellular neurons and dendrites under conditions of increased hormone demand. (A) Arrows delineate the extent of an individual membrane apposition between two SON cell bodies. Compare with figure 55.4A, where glial processes are present between adjacent neurons. Bar = 1  $\mu$ m. (B) Bundles in the dendritic zone of the SON. A small bundle of two dendrites (dendrites numbered with 1's) and a large bundle of six dendrites (dendrites numbered with 2's) are shown. An axon terminal (A) makes synaptic contacts (arrows) with two dendrites, forming a double synapse. Bar = 1  $\mu$ m.

number of dendrites in a given bundle have been found to be significantly greater in prepartum animals than in virgin animals. In the 24 hr between pre- and postparturition, further ultrastructural manifestations of activation are detected. Postpartum animals (2–24 hr after parturition) exhibit a level of dendritic bundling that is higher than that of both virgin and prepartum animals. In addition, preliminary evidence suggests that the size of the entire dendritic zone is increased in postpartum animals compared with that of virgins (Taubitz, Smithson, & Hatton, 1987).

Functionally, the withdrawal of glial processes from between adjacent neural elements may have many different effects. Given that astrocytes act to take up extracellular potassium ( $[K^+]_0$ ) (Orkand, 1977), their relative absence from between neural elements may lead to significant local increases in  $[K^+]_0$ . These local increases could slightly depolarize those areas of cells in direct apposition, thus increasing the overall excitability in the SON. Another potentially important result of glial withdrawal may be the formation of electrical fields around groups of neurons similar to those that form around pyramidal cells in the hippocampus (Taylor & Dudek, 1982, 1984a, 1984b). Both these effects may serve as mechanisms by which MNCs can modify their electrical activity when increases in hormone demand occur. Local elevations of  $[K^+]_0$  probably also aid these peptidergic neurons in the production of their secretory products, since increases in protein synthesis have been shown to occur in brain slices incubated in medium containing even slightly elevated potassium concentrations (Lipton & Heimbach, 1977, 1978).

### Synapse Formation

Withdrawal of glial processes may also play a permissive role in what is perhaps the most amazing ultrastructural change that occurs in the hypothalamo-neurohypophysial system. Synapse formation, an uncommon event in the adult animal in response to physiological stimulation, has been shown to occur in both parturient and lactating animals. Evidence for synapse formation is first seen in the dendritic region of the SON in postpartum animals (Perlmutter et al., 1984). These new synapses have a particular arrangement between the dendrites of the SON. They consist of one axon terminal simultaneously forming synaptic contacts with two or more dendrites (see figure 55.6B). Although these shared axodendritic synapses are found in the dendritic region of the virgin's SON, their frequency is higher in postpartum animals compared with either virgins or prepartum animals. Axodendritic double synapses, which reach their maximum frequency in postpartum animals, decline in frequency with lactation (Perlmutter et al., 1984); however, additional forms of synaptic plasticity have been seen to occur in



**Figure 55.7**

Percentages of SON cell bodies and dendrites contacted by double synapses in virgin females around the time of parturition and lactation. Dendritic double synapses were significantly elevated in postpartum animals; somatic double synapses were significantly increased only during lactation. (Data from Hatton & Tweedle, 1982; Perlmutter et al., 1984)

the time between parturition and day 14 of lactation. Vacant or incompletely covered postsynaptic densities, which are found on about 5% of the dendrites in virgins, are virtually nonexistent in the dendritic zones of lactating animals (Tweedle & Hatton, 1986). A reasonable interpretation of this is that the incompletely occupied or vacant densities have become occupied during stimulation. Synapse formation is also apparent in the somatic region of the SON during lactation and again involves the appearance of new shared synapses similar to those seen in the dendritic region in postpartum animals. New synapse formation in the somatic and dendritic regions of the SON during various functional states is shown in figure 55.7. In addition, studies of postsynaptic specializations in the paraventricular nucleus using freeze fracture techniques have revealed the presence of unique annular synapses. These annular postsynaptic structures appear to last throughout the mother's life and are not found in virgin females or male rats (Hatton & Ellisman, 1982).

Each of the forms of synaptic modification discussed above may participate in the general activation of the hypothalamo-neurohypophysial system during parturition and lactation. The disappearance of incompletely covered postsynaptic densities (i.e., the complete occupation of the densities by presynaptic terminals) may allow a given synaptic input to a dendrite to exert a more powerful influence on the dendrite, as postsynaptic densities are presumably the morphological correlate of neurotransmitter receptors and specialized ion channels (for review, see Siekevitz, 1985). Shared syn-

apses may also be an important mechanism that permits oxytocin cells within the SON to fire in such close synchrony. They may accomplish this by either excitatory or inhibitory actions.

#### Ultrastructural Changes in the Neurohypophysis

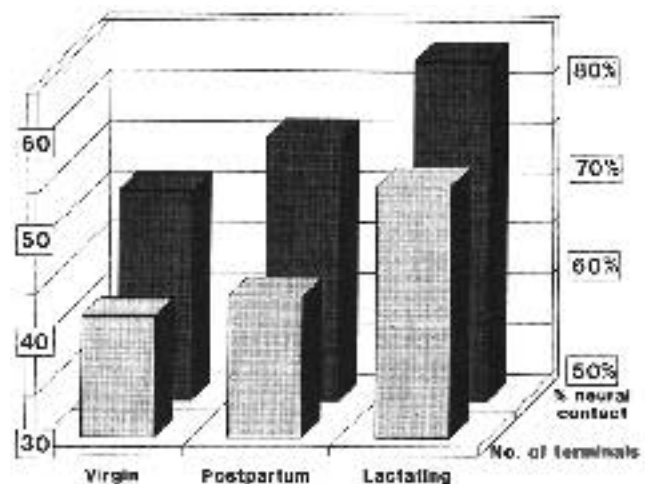
In addition to the striking changes in the region of cell bodies and dendrites of MNCs, ultrastructural reorganization in the neurohypophysis is evident in parturient and lactating animals. As mentioned earlier, axonal profiles in the neural lobe of unstimulated animals are often completely surrounded by pituicyte cytoplasm. The number of enclosed axons varies according to the demand for hormone release; thus the incidence of enclosed axons decreases dramatically in the prepartum–postpartum interval. This decrease in the number of axons completely surrounded by pituicyte cytoplasm remains significantly lower than in either virgin or prepartum animals throughout lactation (Tweedle & Hatton, 1982).

Further restructuring occurs in postpartum and lactating animals at the basal lamina, where terminals secrete their hormone into the circulation (Tweedle & Hatton, 1987). Here the percentage of neural coverage of the basal lamina increases significantly, with a corresponding decrease in pituicyte coverage, in postpartum animals compared with prepartum animals. This increase in neural contact is also maintained throughout lactation. The mechanism by which increases in neural contact occur at the basal lamina appears to differ between parturient and lactating animals. In postpartum animals on the day of parturition, the increased coverage is associated with a dramatic increase in the size of individual terminals, while in lactating animals (10–14 days postpartum), the increased coverage is due to a larger number of terminals in contact with the basal lamina (figure 55.8).

These changes in the arrangements of pituicytes, axons, and the basal lamina may have profound effects on hormone release during parturition and lactation. Axon terminals engulfed by pituicytes are unable to secrete their hormones into the circulation, since they do not contact the basal lamina. The rapid release of enclosed terminals presumably allows a greater number of terminals to contact the basal lamina and participate in effective hormone release. The withdrawal of pituicyte processes from their positions along the basal lamina permits axon terminals greater access to the basal lamina and thus may serve as an additional mechanism that maximizes hormone release.

#### Changes in Neuronal Coupling Associated with Lactation

Detecting alterations in the organization of this system has not been left exclusively to the anatomist. Experiments utilizing hypothalamic brain slices have pro-



**Figure 55.8**

Graph illustrating the temporal relationships between the percentage of neural contact at the basal lamina and the number of terminals per 100  $\mu\text{m}$  of basal lamina in virgin, postpartum, and lactating animals. The scale at the left shows the number of terminals; the scale at the right shows percentage of neural contact. (Data from Tweedle & Hatton, 1987)



**Figure 55.9**

Projection drawing of a pair of dendrodendritically dye-coupled neurons in the SON. An intracellular injection of Lucifer Yellow into one cell resulted in dye transfer to the adjacent cell. These cells have at least one point of contact between their dendrites.

vided further evidence of remodeling in conjunction with increases in hormone demand. The transfer from one neuron to another of an intracellularly injected dye of low molecular weight has long been taken as indirect evidence for electrotonic coupling between cells. One such dye is Lucifer Yellow (Stewart, 1978). Inter-neuronal coupling with this dye was first shown in SON neurons in 1981 (Andrew, MacVicar, Dudek, & Hatton, 1981). An example of dendrodendritic coupling, the predominant type seen in the SON, is shown in a projection drawing in figure 55.9. Recent studies have found that the incidence of dye coupling varied rather predictably with the physiological state of the animal; an increased incidence of dye coupling was presumed to be related to activation of the system.

Thus it has been inferred that electrical coupling among MNCs is another of the plastic properties of this system that is modified according to the functional demands placed on it. Electrical coupling has now been demonstrated directly by recording intracellularly from coupled pairs of SON neurons (Yang & Hatton, 1988).

In addition to the other ways, reviewed above, that the brains of nursing mothers differ from those of untreated virgins, one would expect the chronically increased demand for oxytocin during lactation to produce observable changes in coupling. In two separate studies, it has been found that the incidence of dye coupling among SON cells in lactating rats is more than double that of virgin female animals (Hatton, Yang, & Cobbett, 1987; Yang & Hatton, 1987a). The observed changes were due to an increased incidence of coupling among oxytocin and vasopressin cells, which was interpreted to reflect both the nursing mother's finely tuned oxytocin response to suckling and her ability to respond readily to any challenges to water balance that may arise. It may be that enhanced coupling among oxytocinergic neurons participates in the synchrony of discharge of these cells and/or in metabolic cooperation among them.

In support of a role for electrical coupling among oxytocin cells in mother rats are the results of recent experiments demonstrating direct olfactory inputs to SON cells. Anatomical tracing studies (Hollowell, Smithson, & Hatton, 1986; Smithson, Weiss, & Hatton, 1987; Smithson, Zapp, Hatton, & Weiss, 1988) and electrophysiological studies (Dougherty, Yang, & Hatton, 1986) have shown that afferents from both the main and the accessory olfactory bulbs synapse directly on SON neurons. Electrical stimulation of the output pathway from these two structures is exclusively excitatory on oxytocinergic neurons of the SON. Furthermore, such stimulation for brief periods of time in slices of hypothalamus increases the incidence of dye coupling among SON neurons in tissue from lactating rats. The same stimulation in slices from males or untreated virgin females does not affect the amount of dye coupling observed (Yang & Hatton, 1987b).

It may be that this stimulation is ineffective in normal male and virgin females, in part, because the SON cell bodies, and particularly dendrites of those animals, are separated by interposed glial processes, disallowing formation of junctions. In contrast, these processes have long since retracted from between the neural elements of lactating animals, and thus intercellular junction formation would not be disallowed. Our hypothesis is that the olfactory and vomeronasal stimulation that the nursing mother receives from pup licking and retrieval just prior to suckling serves to increase the electrical coupling among oxytocinergic neurons. This would have the effect of making more efficient

the cellular coordination necessary for the milk-ejection reflex.

### Implications of Changes in Brain Morphology

In addition to its well-characterized role in parturition and lactation, oxytocin may also participate in the expression of maternal behavior. Increased bundling among SON dendrites, similar to that seen in lactating nursing mothers, has been observed in virgin females induced by continuous exposure to young pups to engage in full maternal behavior (Salm, Modney, & Hatton, 1988). Intracerebroventricular injections of oxytocin result in similar ultrastructural changes in the SON (Theodosis, Montagness, Rodriguez, Vincent, & Poulain, 1986) and produce rapid onset of full maternal behavior in virgin animals (Pedersen, Ascher, Monroe, & Prange, 1982). Correspondingly, electrical stimulation of the olfactory pathways in slices of hypothalamus from maternally behaving virgins increases the coupling among SON neurons, just as it does in tissue from nursing mothers (Modney, Yang, & Hatton, 1987). An interaction between olfaction and oxytocin's ability to induce full maternal behavior in virgin animals has also been suggested (Wamboldt & Insel, 1987). Thus it appears that some rather profound brain changes are associated with maternal behavior independently of lactation, some of which have been linked to olfactory stimuli and oxytocin.

These extensive alterations in the organization of the hypothalamo-neurohypophyseal system are impressive, since they occur in response to physiological stimuli. Equally impressive is the complete reversibility of most of these changes. That is, when lactation ceases and the requirements for hormone release return to basal levels, the ultrastructural appearance of the SON resembles that seen in virgin animals. Membrane appositions and double synapses (Salm & Hatton, 1986) in the SON of the female disappear 10 days to 1 month postweaning, and vacant postsynaptic densities reappear in the dendritic zone. Axonal profiles are again found to be engulfed by pituitary cytoplasm, and neural coverage of the basal lamina returns to the levels seen in virgin animals. Of all the changes discussed, the only ones that persist, presumably throughout the life of the animal, are the unique annular postsynaptic specializations that form in the paraventricular nucleus.

### Summary

Profound plastic changes in the hypothalamus and posterior pituitary have been found to accompany parturition and subsequent nursing in rats. These changes involve, at specific times, the dendrites, cell bodies,

and axon terminals of the oxytocin- and vasopressin-secreting magnocellular neurons that compose the hypothalamo-neurohypophyseal system. Also participating in the overall response to the change in status from pregnant to nursing mother rat are the astrocytic glia associated with the different parts of these neurons. By the last day of pregnancy, glial processes have withdrawn from between adjacent magnocellular neurons, resulting in direct soma-somatic apposition and bundling of dendrites. At the same time, the axonal endings in the neurohypophysis are released by the astrocytes that normally enclose them, facilitating hormone secretion during parturition and subsequently during lactation. An increase in axodendritic double synapses occurs around the time of parturition, and the novel appearance of axosomatic double synapses occurs during nursing. Despite glial retraction, expansion of the dendritic zone occurs in postpartum animals in the supraoptic nucleus. Electrical coupling (as indicated by intercellular dye transfer) also increases in nursing mothers. It is likely that many of these changes, at least in the lactating mothers, contribute to the synchrony of firing among oxytocin cells that precedes the milk-ejection reflex. All these modifications appear to be completely reversible. There is evidence from earlier work, however, that certain postsynaptic specializations formed during motherhood remain throughout the life of the animal. Some of these plastic changes, now well documented in mothers, are currently also being found in virgins induced by the presence of pups to behave maternally.

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Nursing behavior constitutes an important natural example of a major time-marked change in regionally localized, behaviorally significant sensory inputs. In female rats, differential stimulation is initiated prior to parturition by the attending to and licking of the nipple skin (Roth and Rosenblatt, 1967) which contributes to mammary gland development (Roth and Rosenblatt, 1968). Substantially greater input changes are incurred with the onset of nursing itself, which is initiated within an hour after the first pup is born (Rosenblatt and Lehrman, 1963; Holloway et al., 1980). During the first days postpartum, female Norway rats spend about 80% of their time nursing; nursing time progressively declines to about 25% by day 17 postpartum (Grota and Ader, 1969, 1974). Suckling stimulates neuroendocrine secretions necessary for milk secretion (prolactin) and milk release (oxytocin) (Tucker, 1988; Wakerley et al., 1988), as well as normal nursing behavior, including prolonged quiescence and the upright crouching posture (Stern and Johnson, 1990; Stern and Taylor, 1991; Stern et al., 1992). While the latter is reflexive (Stern, 1990) and while the female sleeps during much of the long daily nursing period (Stern and Levin, 1976; Voloschin and Tramezzani, 1979) there are numerous daily episodes of nursing initiation (Leon et al., 1978) and the lactating female is clearly alert at the onset of each nesting bout until suckling-induced quiescence occurs (Stern and Johnson, 1990; Stern et al., 1992).

A growing body of studies reveals that representational changes in the primary somatosensory cortical field(s) are induced by new tactual experiences in adult mammals (see Jenkins et al., 1990; Merzenich et al., 1990a,b; Recanzone et al., 1992a–c), including rats (e.g., Delacour, 1987; Kossut et al., 1988; Yun et al., 1989; Kossut, 1992; Welker et al., 1992). With heavy differential stimulation by moving or locationally inconstant tactile stimuli in adults, the zone of representation of the engaged skin within the primary somatosensory cortical field differentially expands (Yun et al., 1989; Jenkins et al., 1990; Merzenich et al., 1990a,b; Kossut, 1992). When moving tactile stimuli are applied in the behavior, inverse changes in recep-

tive field (RF) sizes are concomitantly recorded (Jenkins et al., 1990). Thus, the differentially heavily stimulated skin comes to be represented over a larger cortical territory, and when the inputs are from non-stationary sources, in finer grain.

It is important to note that these positive changes arise only with attended behaviors, and that different changes result from associated inputs as compared with nonassociated inputs (Disterhoft et al., 1975; Woody and Engel, 1975; Weinberger and Diamond, 1988; Ahissar et al., 1992; Recanzone et al., 1992c,d). Moreover, when stimuli are delivered to rigidly fixed skin locations as compared with nonstationary skin locations, RFs can enlarge rather than contract in size (see Recanzone et al., 1992a–c).

It is argued that these changes (Merzenich, 1990a,b; Recanzone et al., 1992c,d; Merzenich and Sameshima, 1993) parallel and account for emergent improvements in discriminative abilities. On the basis of these and many other observations, it has been concluded that the shaping of the details of cortical representations by new and constantly proportionately changing inputs occurs throughout life, with experiences thereby contributing a dynamic neurology to continually evolving behavioral capabilities and perceptions (Merzenich et al., 1984, 1988, 1990a,b; Edelman, 1987).

The major sensory input changes that occur with the initiation of nursing behavior represent a natural epoch in which cortical representational changes should be induced. Those induced changes, in turn, would be expected to contribute to this remarkable, rapidly evolving behavior. These present studies are designed to initiate the investigation of the neurological changes induced in one presumptively involved forebrain region, the “primary” skin surface representation in cortical area SI. Such studies are a starting point for reconstructing the panoply of distributed adaptive neurological changes that are hypothetically driven by—and account for the adaptive neurological aspects of—this complex behavior.

These experiments reveal that significant changes in the body surface representation are produced by nursing behavior. Those changes appear to arise from the

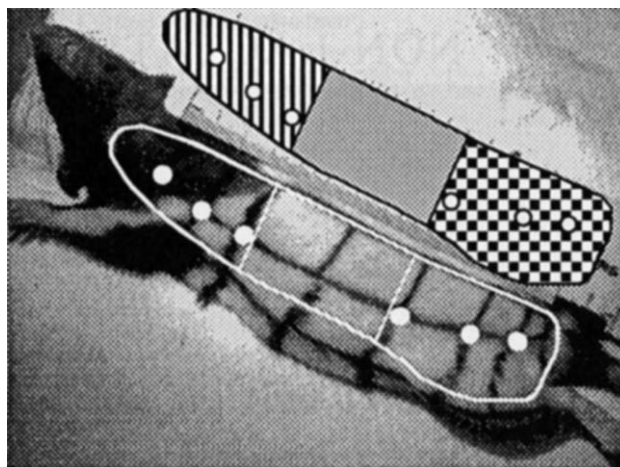
heavy stimulation of the nipple-bearing skin of the trunk ventrum that is generated in the behavior. These representational changes in SI constitute a hitherto undescribed contributor to this complexly emergent behavior.

### Materials and Methods

Reported results are from 13 young adult female Long-Evans rats (*Rattus norvegicus*) weighing 220–360 gm (Simenson Laboratories, Gilroy, CA). Three control rats were virgins, approximately age matched with members of other rat groups. The other 10 females were primiparous, with cortical maps derived at different times after parturition. For four of these rats, designated “nonlactating,” pups were removed from the mother on the day of birth and killed by an overdose of halothane gas. In these four controls, the cortex was mapped at days 12, 14, 16, and 18 postpartum. For five “lactating” mothers, nursing between 9 and 13 pups (mean = 10.6), nursing behavior was unperturbed until the time of initiation of the cortical maps, which were derived at days 6, 10, 12, 16, and 19 postpartum. At those times, pups from these lactating females were killed as described above. In one additional demonstration rat, the cortex was mapped in a primiparous female 2.5 months after weaning following a 2-week-long nursing period. The SI cortical maps derived in three of these 13 rats—one virgin, the +6 d postpartum dam, and the postweaning female—were incomplete, although many data samples were derived in all three cases. Unless otherwise noted, the illustrated data are from the 10 rats in which cortical maps of the nipple-bearing ventrum skin were believed to be complete.

Treatment of rats was within the National Institutes of Health *Guide for Care and Use of Laboratory Animals* (revised 1987). Rats were initially anesthetized with halothane, and then intubated and anesthetized with sodium pentobarbital (50 mg/kg, i.v., for controls; 65 mg/kg for lactating dams). Anesthetic state was monitored by eye-blink and paw-withdrawal reflexes, and supplemental doses of diluted sodium pentobarbital were intravenously administered throughout the course of the experiments to maintain animals at an areflexive level of anesthesia. The rats were given a single dose of atropine sulfate (0.1 mg/kg, i.m.) to suppress respiratory secretions. Body temperature was monitored and maintained between 36°C and 38°C. Rats were killed at the end of each recording experiment by administration of an overdose of sodium pentobarbital.

Long hairs on the trunk were trimmed to facilitate the subsequent estimation of cutaneous RF boundaries. After marking the body surface with indelible grid

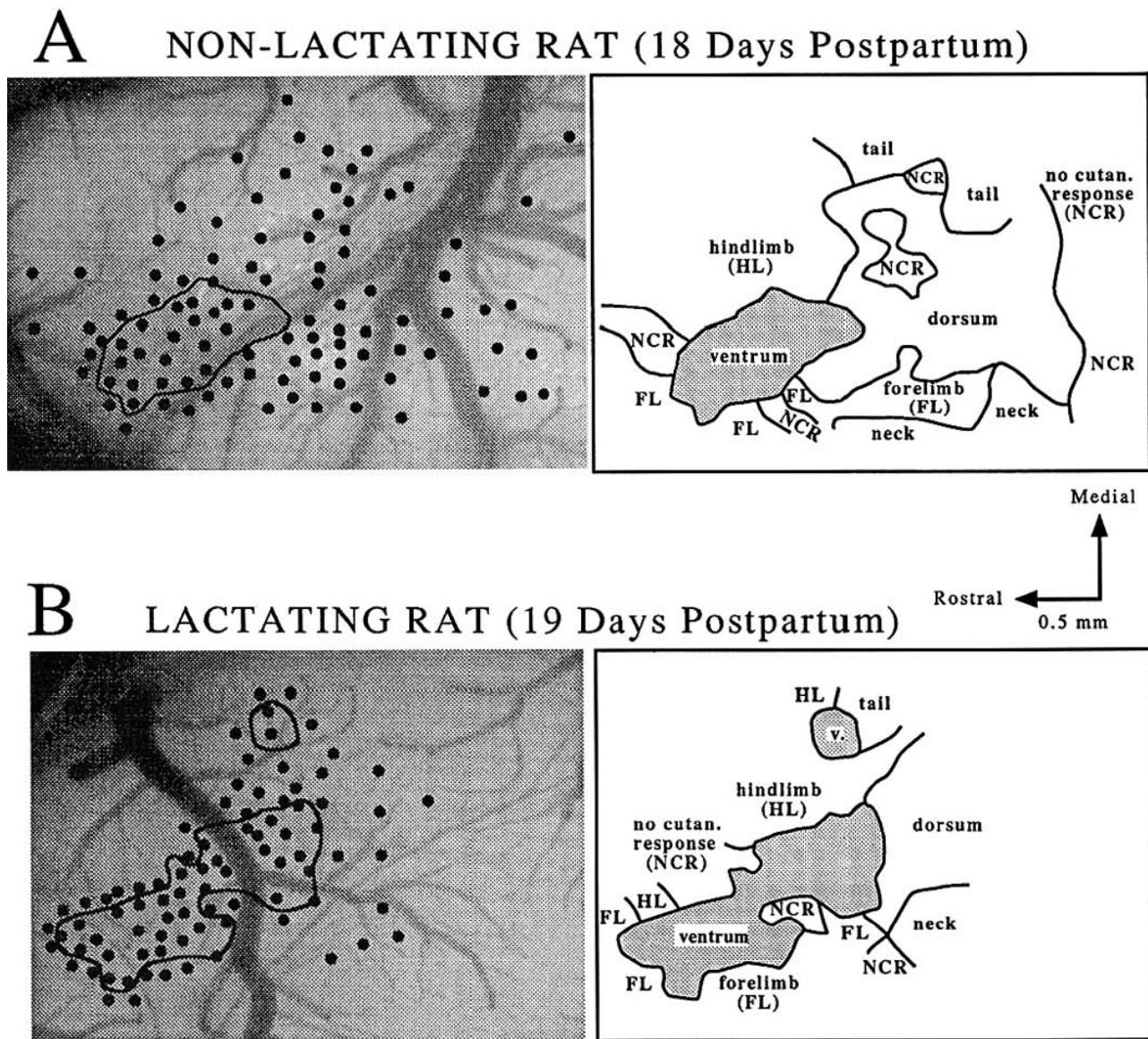


**Figure 56.1**

The skin designated as the “ventrum” of the female rat is outlined in white in this typical computer image taken as part of a cortical mapping experiment. Grid lines marked by indelible ink were used to facilitate the accurate drawing of cutaneous RFs. In the analyses described in Results, the ventrum skin was separated into thirds, as indicated. The anterior and posterior each bear three of the six nipples (white dots) on the mapped side of the ventrum. Ventrum designations in the drawing above match those used in the reconstructions of the ventra in 10 female rats, in figure 56.3.

lines connecting the nipples or oriented orthogonal to them, the surfaces of the trunk contralateral to the exposed cortex were imaged using a Cohu CCD video camera and framegrabber and IMAGE software, in each experiment (e.g., see figure 56.1). The rat was then mounted in a headholder in a stereotaxic frame that provided easy access to the surfaces of the trunk contralateral to the cerebral hemisphere to be mapped. After tracheal cannulation and cerebrospinal fluid drainage through an opening in the foramen magnum, the somatosensory cortex was exposed by a surgical craniotomy. The dura was resected and the directly exposed cortex bathed in a pool of 20 centistoke dimethylpolysiloxane. A 40× computer image of the cortical surface vasculature was then obtained through a Zeiss operating microscope mounted with a beamsplitter and the video camera.

Neuronal responses were recorded from units or small clusters of units by the use of parylene c-insulated tungsten microelectrodes with impedances of about 1.5 MΩ at 1 kHz. At each of 45–135 sample sites, microelectrodes were advanced normal to the cortical surface to a target depth of approximately 750 μm (± about 50 μm). The position of electrode entry into the brain was recorded on the magnified computer image of the brain surface stored by use of multiple-layer CANVAS software, by reference to the details of the cortical surface vasculature. In all experiments, sample sites were separated by about 80–200 μm. Equivalent sampling densities were applied in experimental and

**Figure 56.2**

Mapping and reconstruction procedures used to produce maps of the skin surfaces of the trunk. *Left*, magnified computer image of the surface of a limited sector of the primary somatosensory (SI) cortical field in a +18 d postpartum primiparous nonlactating control rat (*A*), and in a +19 d postpartum primiparous lactating rat (*B*). *Black dots* mark microelectrode penetration sites at which RFs were defined for neurons or small clusters of neurons in the middle cortical layers in the trunk (and surrounding) representational sector(s) of the SI cortex. *Heavy dark lines* outline the estimated zones of representation of the trunk ventra. *Right*, cartoon reconstructions of these two representative mapping experiments. The estimated zones of representation of the ventra are *shaded*. Bordering cortical zones represent the skin of the forelimb (FL), hindlimb (HL), neck, tail, and dorsum. In regions labeled “not cutaneous” (NCR), neurons were not excited by light tactile stimulation, as is typical for significant SI sectors in a zone(s) neighboring the trunk representation (see Welker, 1971; Chapin et al., 1985, 1990).

control cases. All penetrations in any given experiment were parallel.

Receptive fields were defined as those areas of skin at which hair deflection or just-visible skin indentation produced with a fine glass probe evoked a reliable neural discharge, as previously described (Merzenich et al., 1984; Stryker et al., 1987). These skin fields were drawn on the computerized images of the body surface. The grid pattern marked on the fur of the rat with insoluble ink and the mottled colored fur patches on these Long-Evans rats' trunks (figure 56.1) served as guides for accurately drawing RFs on the body surface. The three dimensions of these body surface reconstructions were taken into account at the time of RF definition.

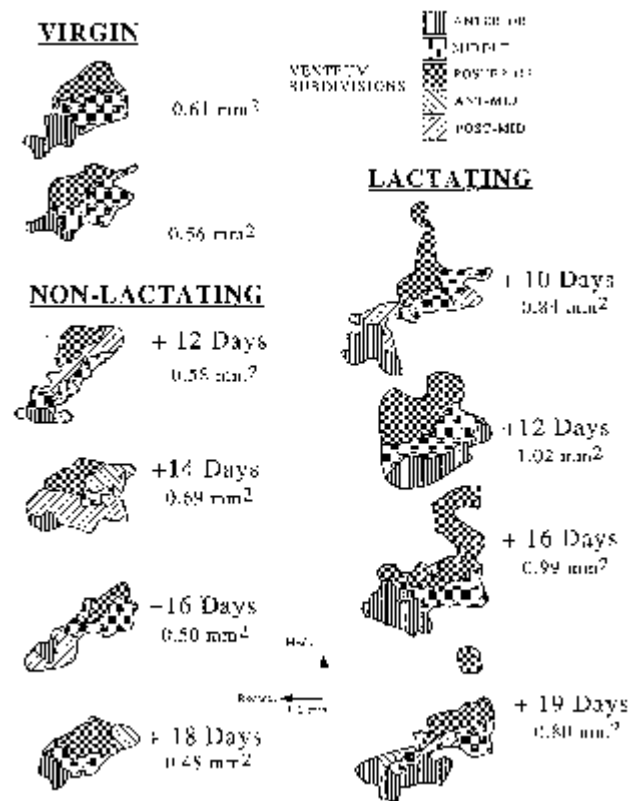
When RFs extended over the nipple skin, it was probed with light tactile stimulation as well as with heavier taps and, in some cases, suction stimuli. In general, as is noted below, low-threshold responses were rarely evoked by nipple or areolar stimulation in these experiments.

### Construction of Cortical "Maps"

Representations of different surfaces of the rat trunk were reconstructed in each experiment. The "ventrum" of the rat was defined as the skin zone extending from the midline to a distance lateral to the nipples equal to half the distance from the midline to the nipple, and included the skin flanking the posteriormost nipples on the inner surface of the thigh. This "ventrum" skin is illustrated by example in figure 56.1. With the development of the mammae in nursing, the total area of the ventrum skin was modestly larger in lactating females ( $59.8 \pm 6.7 \text{ cm}^2$ ) than in nonlactating postpartum controls ( $50.8 \pm 6.1 \text{ cm}^2$ ), and more substantially larger than in approximately age-matched virgin ( $40.3 \pm 3.8 \text{ cm}^2$ ) controls. Approximately proportional differences in overall body weight were recorded between these three roughly age-matched female rat groups.

To reconstruct the cortical representations of the ventrum, the remainder of the trunk skin ("dorsum") and of surrounding body surfaces, cartoon maps were drawn by bounding the cortical sites at which the RFs of neurons were centered on different skin surfaces. Examples of such map reconstructions are shown in figure 56.2.

Cortical representational areas and cutaneous RF areas were determined by the use of measurement software in the program CANVAS (Deneba). RFs were defined by their absolute areas, and with normalization as a function of the total "ventrum" skin surface area measured in each individual rat. Statistical comparisons were performed using an analysis of variance (ANOVA) completed with the Fisher PLSD post hoc test (STATVIEW II).



**Figure 56.3**

Reconstructed ventrum representations from 10 rat cases. Two maps from virgin rats are shown at the upper left; four from postpartum nonlactating rats at the lower left; and four from postpartum lactating rats in the right column. See figure 56.1 and the text for the definition of "ventrum" skin. In ventrum map cartoons, SI representational territories in which RFs were centered on the anterior (vertical stripes), middle (stippled), or posterior (checkerboard) ventrum thirds are distinguished in each drawing. As shown in the key at the top, zones over which neurons had RFs with nearly equal parts on two of these three skin surfaces are indicated by diagonal hatching. In cortical representational areal measures, these zones were divided equally between the bordering skin regions that RFs broadly overlapped. Total ventrum representational areas are shown at the right of each cartoon drawing. Note that the SI ventrum representations were consistently larger in lactating rats than in controls. See text for further details.

### Results

Maps of the representation of the trunk ventrum are shown for representative postpartum lactating and nonlactating female rats in figure 56.2. Penetration sites are shown with reference to the cortical surface vasculature in the panels at the left. Territories of representation of the ventrum skin (see figure 56.1) are outlined. The location of the outlines between sample sites reflects the proportional part of the RF that overlaps the ventrum and other bordering, designated skin surfaces. Note that all reported ventrum maps were bounded by samples in which neurons were predominantly driven by stimulation of other skin surfaces.

In these two typical examples, ventrum representation zones in SI were directly flanked by 22 and 36 penetrations in which RFs were located predominantly on other skin surfaces. With many boundary site definitions as in these cases, a relatively accurate estimation of the territories of representation of the ventrum was obtained (see Stryker et al., 1987).

Cartoon drawings illustrating functionally defined cortical representational zones are shown at the right of each drawing in figure 56.2. The ventrum representation is flanked posteriorly by a large representation of the “dorsum” (lateral and dorsal surfaces) of the trunk. Hindlimb, forelimb, and noncutaneous response zones also commonly bordered the ventrum representation (see Welker, 1971, 1976; Chapin and Lin, 1984, 1990). The actual skin surfaces across ventrum borders and the lengths of these border segments were highly variable in different mapped females in all rat series.

#### Cortical Area of Representation of the Ventrum in Primiparous Lactating versus Primiparous Nonlactating and Virgin Rats

Cartoon drawings outlining the territories of representation of the ventrum are illustrated for 10 rats in figure 56.3. In those drawings, representational territory is subdivided with respect to whether RFs were centered over the anterior third, the middle third, or the posterior third of the trunk ventrum (see figure 56.1 for this tripartite ventrum division; see figure 56.3 key, top right). In figure 56.3, (1) maps from two virgin rats are shown at the upper left, (2) maps from four nonlactating rats are shown in the lower left column, and (3) maps from four lactating rats are shown in the column at the right.

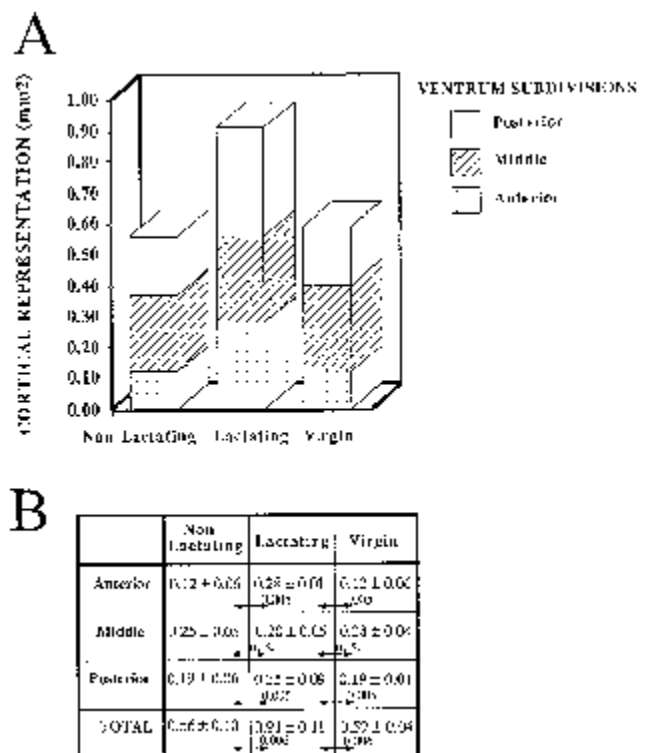
The SI territories of representation of the ventrum for virgin and for roughly age-matched postpartum nonlactating rats were approximately identical in size (mean  $\pm$  SD =  $0.59 \pm 0.04$  vs.  $0.56 \pm 0.10$ ; see table 56.1). On the other hand, the SI territory of representation of the ventrum was, on the average, about  $1.6\times$  larger in lactating than in control rats. This difference between lactating rats versus both virgins and nonlactating primiparous controls was significant at the  $p < 0.006$  level [ $F(2, 7) = 15.43$ ].

When the cortical territories of representation of the anterior and posterior nipple-bearing ventrum thirds and the central third devoid of nipples were distinguished, it was evident that the differences between zones of cortical representation in lactating versus control rats occurred only for the former (figure 56.4). Thus, the representation of the nipple-bearing anterior ventrum was about  $2.3\times$  larger on average than for controls; the nipple-bearing posterior skin was about  $1.8\times$  larger. Differences between lactating rats and

**Table 56.1**  
Cortical representation ( $\text{mm}^2$ )

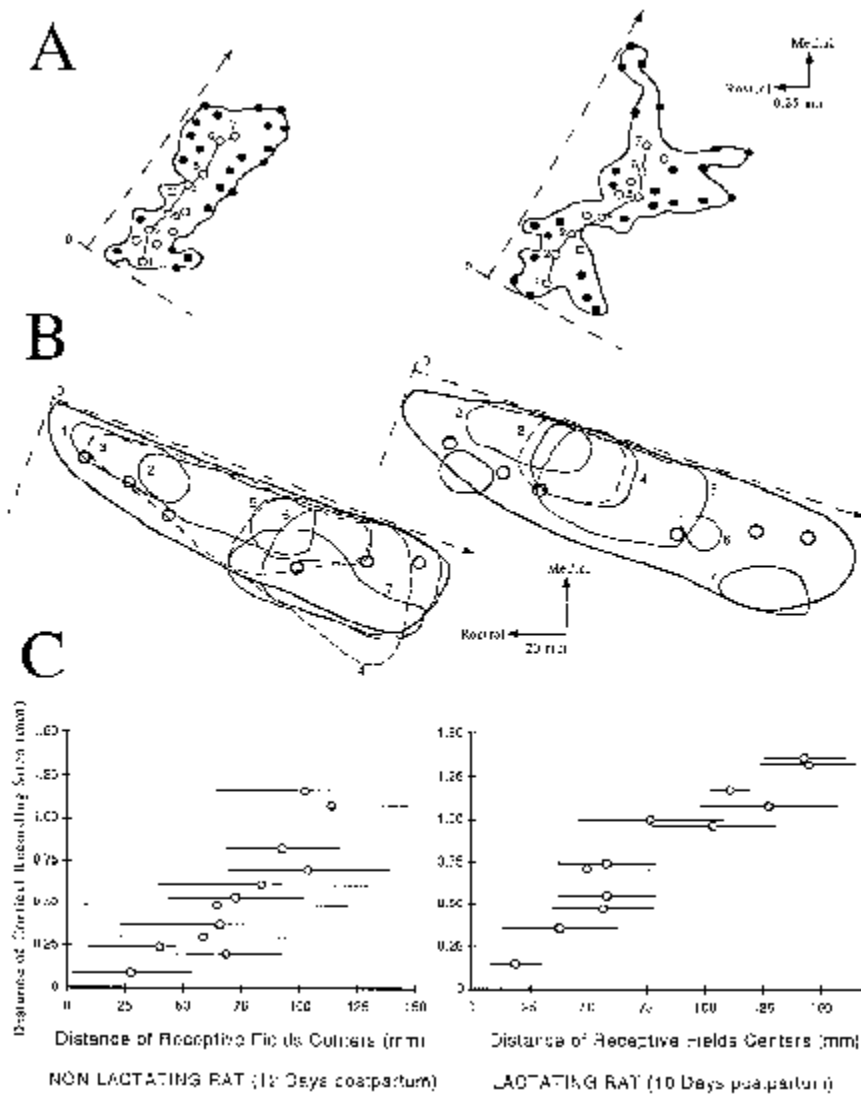
	10 d	12 d	14 d	16 d	18 d	19 d	Mean $\pm$ SD
<b>Nonlactating</b>							
A <sup>a</sup>	—	0.08	0.20	0.12	0.08	—	$0.12 \pm 0.06$
M	—	0.27	0.27	0.28	0.17	—	$0.25 \pm 0.05$
P	—	0.23	0.22	0.10	0.23	—	$0.19 \pm 0.06$
	—	<b>0.58</b>	<b>0.69</b>	<b>0.50</b>	<b>0.48</b>	—	<b><math>0.56 \pm 0.10</math></b>
<b>Lactating</b>							
A	0.29	0.28	—	0.26	—	0.28	$0.28 \pm 0.01$
M	0.30	0.32	—	0.29	—	0.21	$0.28 \pm 0.05$
P	0.25	0.42	—	0.44	—	0.31	$0.35 \pm 0.09$
	<b>0.84</b>	<b>1.02</b>	—	<b>0.99</b>	—	<b>0.80</b>	<b><math>0.91 \pm 0.11</math></b>
<b>Virgin</b>							
A	0.16	0.07	—	—	—	—	$0.12 \pm 0.06$
M	0.25	0.31	—	—	—	—	$0.28 \pm 0.04$
P	0.20	0.18	—	—	—	—	$0.19 \pm 0.01$
	<b>0.16</b>	<b>0.56</b>	—	—	—	—	<b><math>0.59 \pm 0.04</math></b>

<sup>a</sup>Divisions of ventrum: A, anterior; M, medial; P, posterior (see figure 56.1).



**Figure 56.4**

A, Mean areas of representation of the posterior, middle, anterior, and total ventrum surfaces for postpartum nonlactating, lactating, and age-matched virgin rats. B, Group (mean  $\pm$  SD) statistics for these three experimental series. Significance levels are indicated by arrows; *n.s.*, no significant difference.



**Figure 56.5**

Representative RF sequences from maps derived in postpartum nonlactating (*left*) and lactating (*right*) rats. Note that only a fraction of the cortical response sampling sites for these maps are indicated in these cartoons. As penetration sites shifted across the SI ventrum representational zone (*A*), RFs almost always shifted in an orderly, continuous shifted-overlap progression across the rat ventrum (*B*), in all experimental cases. In *C*, the rostrocaudal extent of each RF is represented by a *horizontal bar* to more clearly show the extents of RF overlaps in this typical RF progression.

all controls were significant at the  $p < 0.005$  level [ $F(2, 27) = 8.85$ ]. By contrast, while larger on the average in these small series, the SI territories of representation of the nipple-devoid middle third of the ventrum (see the inset, figure 56.3) in lactating rats did not differ significantly from that recorded in either control group (figure 56.4B).

#### Evidence for Continuous Topographic Expansion of a Shifting-Overlap Representation of the Ventrum in Lactating Rats

The internal shifting-overlap topographic order of RFs representing the ventrum skin was not obviously differ-

ent in lactating females as compared with control rats (figure 56.5). That is, RFs generally shifted continuously as penetrations moved across the cortex in any direction, as in the representative examples in figure 56.5, with the representation of the extreme margins of the ventrum skin located at the extreme margins of the SI ventrum zone. For example, RFs representing the skin along the midline were invariably arrayed along the far rostral border of the SI ventrum zone, RFs bordering the more dorsal trunk were invariably represented caudolaterally, the skin over the anterior trunk in front of the forelegs was represented at the anterior extreme of the ventrum zone, and the perivaginal

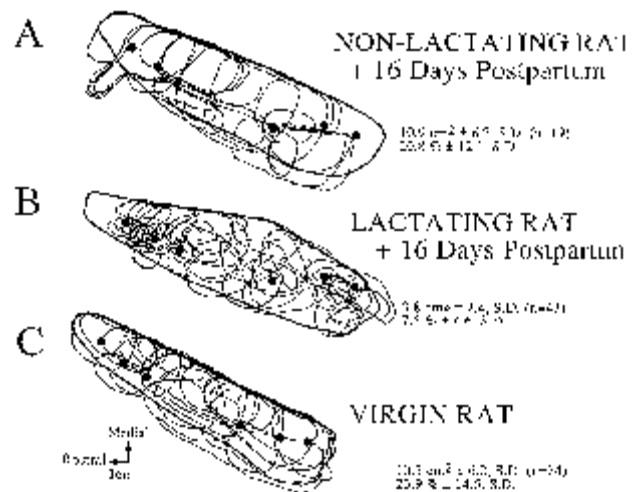
skin was represented in the extreme medial or rostro-medial zone. Thus, in its apparently larger form in lactating females, there was still a simple, single, orderly representation of the ventrum skin in SI.

As the ventrum representation enlarged, what other skin surfaces likely lost SI representational territory? Chronic recording experiments in rats carried through parturition and nursing would have to be conducted to resolve this issue with certainty. However, examination of bordering skin surfaces in both control groups and in nursing rats indicates that in different rats, zones of representation of the nipple-bearing ventrum zones between the forelegs and hindlegs border the zones of representation of forelimb and hindlimb skin, as well as the zone of representation of the trunk dorsum, and an SI zone(s) in which responses were not cutaneous. With representational expansion apparently consequent from suckling, ventrum representations in some rats appear to widen, others appear to elongate, or both changes occur. Encroachment of the expanding representation across all of its bordering representational zones would appear to be possible.

It has long been argued that there is a general correspondence between SI layer 4 "anatomical maps" of the body surface of the rat and the physiologically defined body surface specificity of recorded neuronal responses (see Welker, 1976). The present results indicate either that nursing-induced changes create a discordance between layer 4 "anatomical maps" of the body surface and functionally defined maps of these skin surfaces, or, alternatively, that a parallel change in the layer 4 "anatomical map" is induced by this behavior. Directly correlated anatomical and physiological studies must be conducted to resolve which of these interesting possibilities actually applies.

#### Sizes and Distributions of RFs in Lactating versus Control Rats

RFs reconstructed from neuronal response samples in the ventrum representational zone in lactating rats were smaller than were those recorded in control populations. All ventrum RFs from a representative cortical map taken from each of these short series are shown in figure 56.6. There, the ventrum skin is outlined by a heavy black line; nipples are indicated by small dark circles; cutaneous receptive fields are outlined by thinner black lines. As in all such cases, differences in the sizes and regional distributions of RFs are evident at a glance. In these representative cases, the absolute skin areas of RFs averaged 10–11 cm<sup>2</sup> in both control maps (figure 56.6A,C), while they averaged 3.8 cm<sup>2</sup> in the lactating rat (figure 56.6B). Moreover, small RFs were recorded in larger numbers in the more anterior and more posterior aspects of the ventrum skin in the lactating case.



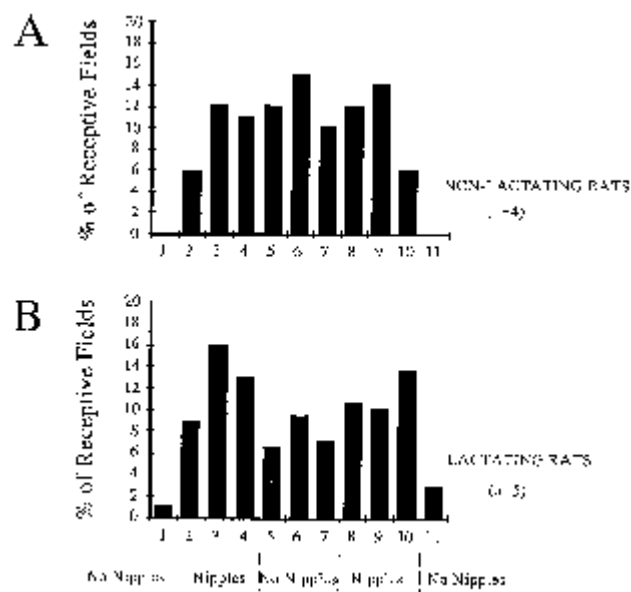
**Figure 56.6**

All RFs defined in the SI ventrum representational zone in a representative postpartum nonlactating rat (A), lactating rat (B), and virgin rat (C). The heavy dark lines outline the ventrum skin; small solid circles mark the locations of nipples; thinner lines outline cortically recorded cutaneous receptive fields. The means and SDs of measured and normalized (with reference to total ventrum skin area) RF sizes are shown for each case, at the right.

The difference in the representation of the anterior and posterior nipple-bearing skin regions as compared with the nipple-devoid central ventrum sector is illustrated in another way in figure 56.7. There, the numbers of RF samples were sorted as a function of the anterior-posterior position of their RF centers, for five lactating and for four postpartum nonlactating control rats. There was a clear bias for the representation of nipple-bearing skin between the hindlimbs and forelimbs of lactating females, but not in nonlactating controls. Dispersions of samples across the skin in virgin rats did not obviously differ from those in postpartum nonlactating rats.

When ventrum RF area data were compared for these different small female rat populations (figure 56.8A, table 56.2), on average: (1) RFs were 2.3× smaller in lactating [ $4.88 \pm 3.33$  (SD) cm<sup>2</sup>] than in virgin rats [ $11.45 \pm 6.16$  cm<sup>2</sup>] [ $F(2, 329) = 101.99$ ,  $p < 0.00001$ ], (2) RFs were 2.8× smaller in lactating than in approximately matched nonlactating postpartum females [ $13.92 \pm 7.08$  cm<sup>2</sup>,  $p < 0.00001$ ], while (3) areas of RFs were smaller in virgin than in nonlactating controls ( $p < 0.05$ ).

It was noted earlier that with the development of the mammary, the overall surface area of the ventrum was modestly greater in lactating than in nonlactating rats, and was greater in both lactating and nonlactating rats than in virgin controls. To compensate for these differences in physical skin expansion, RF data for the three rat populations were normalized by reference to the total measured ventrum skin surface in each



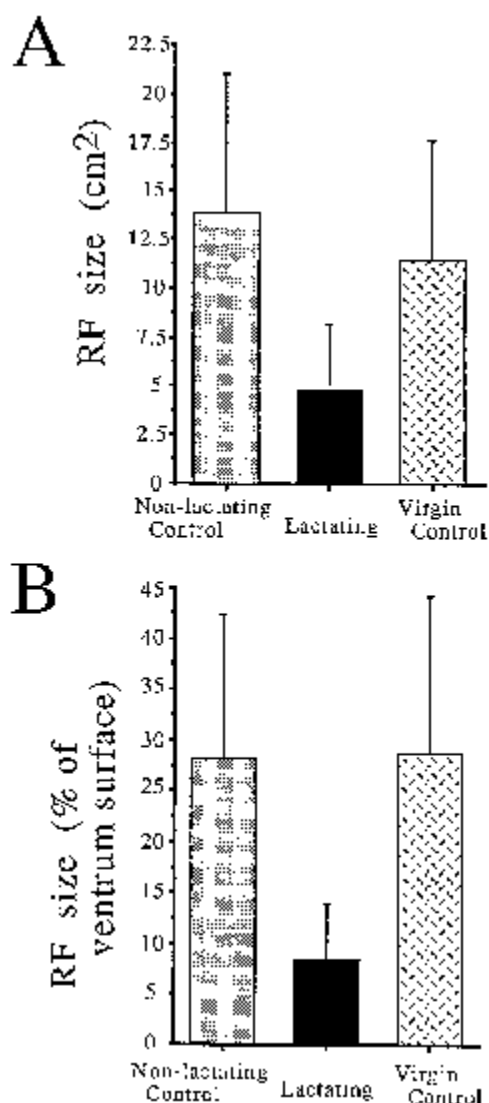
**Figure 56.7**

Percentage of all recorded SI ventrum RFs sorted as a function of the locations of RF centers along the rostrocaudal dimension of the ventrum skin. Data are from four nonlactating postpartum control rats (A) and from five postpartum lactating rats (including the +6 d rat) (B).

individual, as shown in figure 56.8B. With normalization, differences in RF areas in lactating versus control populations were even greater ( $3.3\times$ ;  $p < 0.00001$ ). On average, RFs in lactating rats occupied 8.5% of the ventrum surface, as compared with 28.1% and 28.6% of the ventrum surface in nonlactating and virgin controls. Note that after normalization, the mean RF sizes and dispersions for the two control groups were virtually identical.

#### Differences in RF Sizes for Anterior, Middle, and Posterior Ventrum Representation Sectors

As with cortical representational area, differences in RF sizes between lactating and control group samples were greater for the nipple-bearing anterior and posterior ventrum representational zones than for the representation of the nipple-devoid middle zone (figure 56.9). RFs in the ventrum skin between both the forelegs and hindlegs of lactating rats were about one-third the size in lactating rats than in nonlactating postpartum rats, while those for the middle third in lactating females were a little more than one-half as large. Differences between RFs from all three ventral zones in lactating rats and controls were significant at the  $p < 0.001$  level [ $F(5, 166) = 33.80$ ]. As is evident, for example, in the RF drawings in figure 56.6, RFs for both the anterior and posterior regions were significantly smaller than were those for the central nipple-devoid skin sector (figure 56.9B).

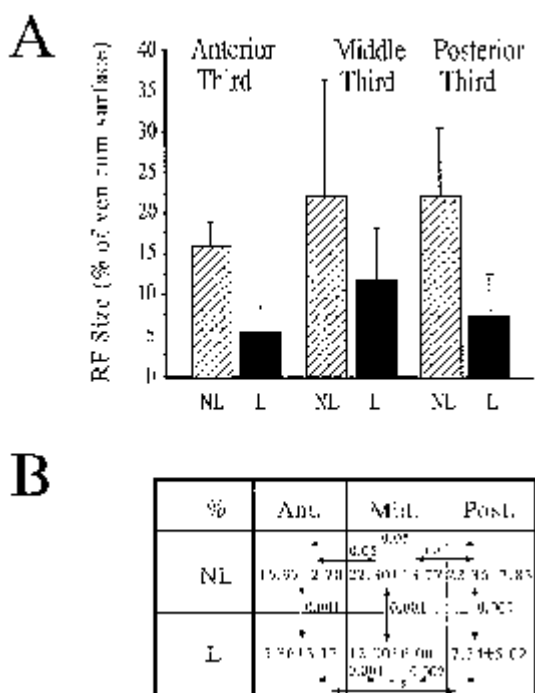


**Figure 56.8**

A, RF size means and SDs from the ventrum trunk representational zones in postpartum nonlactating (left), lactating (center), and virgin (right) rat groups. Data from the +6 d postpartum rat is included in this analysis as it is not significantly different from the data from the other four postpartum rats, and except for the consequences of an increase in  $n$ , its inclusion has no important impacts on these statistics. Error bars are SDs. B, RF sizes for these three populations, normalized with reference to total ventrum skin area.

**Table 56.2**  
Receptive field sizes

	6 d	10 d	12 d	14 d	16 d	18 d	19 d	Total Population
<i>Nonlactating</i>								
cm <sup>2</sup>	—	—	14.86 ± 6.86	12.37 ± 6.79	10.86 ± 6.29	17.56 ± 7.10	—	13.92 ± 7.08
%	—	—	33.76 ± 15.45	25.77 ± 14.15	20.84 ± 12.06	30.23 ± 12.23	—	28.07 ± 14.37
n	—	—	28	29	19	21	—	97
<i>Lactating</i>								
cm <sup>2</sup>	3.59 ± 2.00	7.92 ± 4.70	4.20 ± 1.62	—	3.81 ± 2.36	—	5.29 ± 3.25	4.88 ± 3.33
%	7.99 ± 4.44	12.19 ± 7.24	7.27 ± 2.81	—	7.47 ± 4.63	—	8.04 ± 4.94	8.54 ± 5.28
n	36	32	27	—	43	—	34	172
<i>Virgin</i>								
cm <sup>2</sup>	10.27 ± 6.21	11.02 ± 5.41	12.76 ± 7.14	—	—	—	—	11.45 ± 6.16
%	23.94 ± 14.48	30.61 ± 15.04	30.46 ± 17.04	—	—	—	—	28.65 ± 15.45
n	24	23	18	—	—	—	—	65

**Figure 56.9**

**A**, Normalized RF areas centered over the anterior, middle, and posterior thirds of the ventrum. Normalization was with respect to the measured total area of the ventrum surface. Data are from postpartum nonlactating (NL) rat series (cross-hatched), and lactating (L) rats (solid). RFs that extensively overlapped two or occasionally three of the skin zones were not included in the analysis. **B**, Statistical summary of RF area differences between lactating and postpartum nonlactating rats.

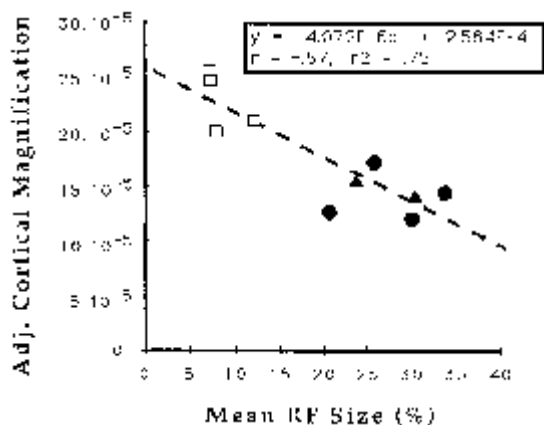
### Relationships between Cortical Magnification and RF Sizes

In some other examples of cortical remodeling with experience or after peripheral lesions, cortical magnification (cortical area/skin surface area) appeared to increase as an approximately inverse function of the change in RF sizes (see Merzenich et al., 1984, 1985; Jenkins et al., 1990) suggesting that a “hypercolumn rule” might be actively maintained in the cortex (see Hubel and Wiesel, 1974; Sur et al., 1985). In accordance with a hypothetical “hypercolumn rule,” any given RF would be represented over an approximately constant-sized cortical area (a cortical “hypercolumn”), regardless of RF size.

In plotting cortical magnification against RF size in the present experiments, it is necessary to compensate for the ballooning of the skin accompanying mammary development in the estimation of both cortical magnification and RF size (figure 56.10). With compensation for this skin expansion, an inverse relationship with an approximate proportion of 3:2 between cutaneous RF sizes and cortical magnifications was recorded. That is, although cortical magnification and RF size appeared to be systematically related, a strict “hypercolumn rule” requiring a 1:1 proportionality was *not* maintained during these representational changes. This observation—especially taken with results in studies of Recanzone et al. (1992b) in which RF changes were generated that are proportionally *greater* than cortical areal representational changes—indicates that a cortical magnification/RF size “hypercolumn rule” is not strictly maintained during representational remodeling by the cortical machine, as was earlier hypothesized (see Merzenich, 1983, 1984a,b).

### Nonexistent or Limited Representation of the Nipple and Areolar Skin in SI Cortex

RFs often completely encircled one (occasionally, more than one) nipple(s). The skin surfaces on the nipples of



**Figure 56.10**

SI cortical magnification (= cortical representational area/adjusted ventrum skin area) plotted as a function of the mean normalized RF size. *Open squares* represent lactating female cases; *solid circles* are from nonlactating controls; *solid triangles* are from virgin rats. The *dashed line* is the best-fit straight line. Note that in defining cortical magnification, the stretching of the ventrum skin, largely due to the development of the underlying mammary glands, is compensated for by using measures of the nonexpanded ventrum skin from matched virgin controls, for all three experimental groups. Mean RF areas were also normalized with reference to the total ventrum area. See the text for a justification of these adjustments.

lactating rats are substantial, as they extend to lengths of more than a centimeter in suckling. Moreover, the glabrous areolar skin surrounding each nipple was relatively large in nursing females, especially when compared with virgin controls. When RFs extended across the hairless skin of the nipple and areola, those skin surfaces were explored with light tactile stimulation. With a handful of possible exceptions from more than 100 such samples, responses are *not* evoked from this skin by light tactile stimulation, while single units at these same cortical sites were commonly exquisitely sensitive to deflection of the hairs surrounding the areolar skin.

It might be noted that while we also applied heavier stimuli including nipple suction in mapping RFs in several rats, we did not apply such stimuli consistently, and we did not investigate responsiveness of these neurons to suckling per se. In fact, our neuron sample would be excited by suckling pups, but on the basis of the excitation of sensitive hair receptor responses from the fur surrounding the nipple, given the muzzle contact and the treading of pups on the nearby skin (Wakerley and Drewett, 1975). In practice, any specific inputs evoked from glabrous skin during normal suckling would be difficult to distinguish from those derived from the surrounding hairy skin. In any event, in the small number of samples in which artificial vigorous suction stimuli were applied exclusively to extended nipples, no strong neuronal responses were evoked in this SI ventrum representational zone.

### Two Special Controls: Early Postlactation, Late Postweaning

Two additional special cases were studied in detail in this series. One lactating rat was examined 6 d after initiation of lactation, and a second, 2.5 months after weaning that followed a 2-week-long nursing period. Unfortunately, the ventrum skin was not completely mapped in either individual, so no certain conclusions can be drawn about the SI territories of representation of the ventrum in these cases. However, a large RF sample in both cases revealed that (1) RFs in the ventrum zone of the +6 d postpartum female had a mean area of 3.36 cm<sup>2</sup> (8% of the ventrum surface), which closely matched those recorded for the other longer term lactating rats in this series; (2) by contrast, RFs in the postweaning rat had a mean area of 9.0 cm<sup>2</sup> (16.8% of the ventrum), which approached that of control groups. These results indicate that major RF changes have emerged by 6 d after nursing onset, and that at least this aspect of nursing-induced changes reversed to approach the condition of control rats after a 2.5-month-long weaning period.

### Representation of the Trunk Dorsum in Lactating versus Control Rats

The dorsum was completely mapped in two rats in each of the three experimental groups. Dorsum RFs were significantly smaller in lactating and nonlactating rats than in virgins. At the same time, no significant differences in dorsum RF sizes were recorded between postpartum nonlactating controls and lactating females.

### Discussion

With the onset of suckling, the ventral skin surfaces of a lactating female rat are subjected to a heavy schedule of differential skin stimulation. These experiments indicate that as an apparent consequence, the SI representation of the nipple-bearing skin of the anterior and posterior ventrum in the Norway rat expands about twofold in area for cortical maps derived 10–19 d after nursing onset. In parallel, RFs representing these differentially stimulated skin surfaces are reduced to about one-third of their control sizes. Thus, the regions of the ventrum skin surfaces surrounding the nipples that are most heavily stimulated in suckling come to be represented in finer topographic grain. These cortical representational differences are plausibly related to gains in sensitivity, sensation magnitudes, and tactile acuity (see Merzenich et al., 1990a,b; Recanzone et al., 1992c,d; W. M. Jenkins and M. M. Merzenich, unpublished observations).

We conclude that the initiation of this epochal regional change in skin inputs results in the emergence of central representational changes that presumably become an intrinsic part of nursing behavior. By that

view, this complex behavior is initiated mainly reflexly (Stern, 1990, 1991; Stern and Johnson, 1990; Stern et al., 1992), but with reorganization of central representations of the ventrum induced by the behavior, an overlay of adaptive changes consequent from suckling, treading, and other related stimulation evolves that contribute to the sensations and perceptions that are a main aspect of nursing. Changes in SI probably also occur in the zone of representation of the snout and vibrissae, inputs from which have also been demonstrated to be important for aspects of maternal behavior (Kenyon et al., 1981, 1983; Stern and Kolunie, 1989, 1991; Morgan et al., 1992).

It should be emphasized that these SI changes are likely paralleled by more dramatic representational changes that apply to the central representation(s) of the nipple and areolae. Their skin is not well represented—clearly, not predominantly represented—in the SI cortical field. It has been demonstrated that afferent inputs from the glabrous skin of the nipples and areolae pass into the lateral columns and not the dorsal columns that ultimately provide the main inputs to SI cortex (Fukuoka et al., 1984; Dubois-Dauphin et al., 1985; Poulain and Wakerley, 1986; Tasker et al., 1986; Wakerley et al., 1988). The cortical destination(s) of this input is unknown. Whatever its subcortical and cortical terminus, it is highly likely that nipple/areolar representations undergo a representational transformation as a consequence of the dramatic differences in afferent inputs from the nipples that result from the onset of suckling.

### Some Methodological Precautions

**Possible Contributions of Pre- and Postnatal Changes in Skin Mechanics and Receptor Sensitivity** In humans, there is an almost immediate postpartum increase in the sensitivity of both the glabrous and hairy skin of the breast, regardless of whether or not breast feeding occurred, that is argued to be due to the dramatic decline in hormone levels at parturition (Robinson and Short, 1977). These sensitivity changes progress for at least 4 d following nursing onset; skin sensitivity over longer postpartum times has not been examined. Whether “immediate” hypothetically hormonal effects are direct or only reflect changes in skin mechanics is unknown. Changes in the sensitivity and the dynamic ranges of skin afferents would be a possible consequence of increased skin tension and resistance, for example, as the breasts become engorged (Vorheer, 1974), and such differences as well as possible direct hormonal effects on skin mechanics and skin receptors (see Kow and Pfaff, 1973; Adler et al., 1977; Bereiter and Barker, 1980; Bereiter et al., 1980) might well contribute to the very rapid remodeling of cortical repre-

sentations that follow. On the other hand, (1) no very obvious differences in skin sensitivity or response magnitudes were recorded on the ventrum of lactating rats as compared with nonlactating or virgin controls; (2) the hairy skin over the mammae of lactating females was actually relatively flaccid; (3) hormonal effects in nonlactating rats are identical to those in lactating rats through the time of parturition, and the skin of postpartum nonlactating females expanded significantly, while cortical representations and relative RF sizes in this nonlactating postpartum control population were virtually indistinguishable from those in matched virgin rats; (4) if RF differences were attributable to changes in response sensitivity alone, then RFs would be expected to be larger in size in the more sensitive lactating females—not smaller—as is the case; (5) peripheral estrogen effects are interpreted to apply generally to all body skin (Bereiter and Barker, 1975, 1980; Bereiter et al., 1980)—in our experiments, differences in cutaneous RF sizes were significant only for the ventrum skin; RFs on the flank and dorsum of the trunk were not different in lactating and control rats; (6) no mere change in afferent sensitivity alone can account for a topographically ordered representational expansion necessitating a point-by-point redefinition of RFs for cortical neurons all across the ventrum SI representational zone.

Thus, while changes in receptor sensitivity may contribute to the rapidity and magnitude of these changes, cortical representational remodeling driven by differentially heavy stimulation of the skin overlying the mammary glands is the probable primary cause of them.

**Increase of Ventrum Skin Area** With prepartum body weight gains in pregnancy, and with the postpartum development of the mammae, the ventrum skin surface expands in area by about 50%. How does this skin expansion relate to the representational changes recorded in the cortex? We can assume that afferents continue to innervate the same hair follicles over a dimensionally expansible skin surface. In that case, skin expansion alone would be expected to result in proportionally larger—not smaller—central RFs. Such “expansion” also occurs in nonlactating postpartum control females. In any event, when RFs were normalized with reference to the ventrum skin surface area, RFs in those control females were indistinguishable from those in virgin controls, while before this correction for skin expansion, they were significantly larger, in exact proportion to the ballooning of their ventra.

While the expansion of the ventrum skin in lactating rats is paralleled by an increase in its territory of representation in the cortex, this ballooning of the skin does not appear to be the main dependent variable underlying cortical representational enlargement, as: (1) There

was also a significant expansion of the ventrum skin of postpartum nonlactating controls, but without nursing, no parallel SI cortical representational change occurred. (2) The skin over the central third of the ventrum appears to balloon as much as does the skin in the posterior and anterior thirds, yet representational changes for this sector did not differ significantly from controls with smaller ventra, and RF changes were modest in this region when compared to those for nipple-bearing skin zones. (3) Basic innervation patterns of the skin should be proportionately constant throughout skin expansion. The expanded ventrum skin in lactating females should be innervated by a set of first-order skin afferents that is identical to those that innervated the same (then nonexpanded) skin before pregnancy.

**Limitations of These Experimental Cortical Mapping Procedures** We have earlier analyzed the magnitudes of errors for estimating representational territories by these mapping and reconstruction methods (see Stryker et al., 1987). The errors in estimating territories of representation are a function of sample densities and the numbers of sites at which representational boundaries are defined. Given the grain of sampling and the numbers of samples within the ventrum zone achieved in the present experiments, there is a less than 10% error for estimations of SI ventrum zones in these cases.

RFs were defined partly on the basis of subjective response criteria, and often by recording from multiple unit clusters. In validation of these procedures: (1) Very equivalent RF sizes and dispersions were defined by different experimentors in the same rat experiments, and by different experimentors who defined receptive fields in different rat members of the same experimental group. (2) When single units were discriminable in the record, their RFs were usually little different from those of other units in the "cluster." That is a general finding for recording in deep layer 3 (see Stryker et al., 1987; Chapin and Lin, 1990). (3) Recording conditions were identical in lactating and control rat series. (4) Proportionate differences defined in representational areas and RF sizes in the anterior, middle, and posterior thirds of the ventrum were not recognized until after all experiments were completed. The subsequent revelation of these highly consistent regional differences validates the repeatability and accuracy of RFs localization and delimitation procedures.

#### **Differential Changes for the Representation of Nipple-Bearing Ventrums**

Rat pups spend the great majority of their time centered on the nipples, and provide much weaker excitation of the midventrum nipple-free region (Stern et al., 1992). In fact, that skin is commonly folded and is less

accessible in the typical high upright, crouched position (Stern and Johnson, 1990). Even when the dam is lying down while nursing, the pups are on the nipples. The disproportionate consequences for the representations of the nipple-bearing skin between the forelegs and hindlegs may simply reflect differences in the overall weights of inputs generated from these different skin surfaces in nursing behavior. It is also possible that these representational differences for nipple-bearing and non-nipple-bearing ventrum skin are exaggerated by the fact that cortical plasticity is dependent upon the behavioral significance of inputs (Disterhoft et al., 1975; Woody and Engel, 1975; Weinberger and Diamond 1988; Jenkins et al., 1990; Ahissar et al., 1992; Recanzone et al., 1992c,d). That is, if the dam selectively attends to inputs that are associated with suckling, that might further amplify their specific effects for generating representational change in SI.

#### **Some Earlier Studies on a Possible Cortical Role in Nursing Behavior**

Beach (1937) found that large lesions of the neocortex are detrimental to performance of maternal behavior. Lesions to the anterior half of the neocortex generated greater deficits than did more posterior lesions. At the same time, the degree of impairment was more strongly related to lesion size than to its specific location. In later studies, investigators focused on the consequences of inducing restricted limbic system lesions, including cingulate cortex lesions (for reviews, see Slotnick, 1967; Numan, 1988; Stern, 1989). Among many other findings, these studies demonstrated a probable role of the medial preoptic nucleus in the regulation of maternal behavior (Numan, 1988), and demonstrated connections of this nucleus with the SI cortical field (Simerly and Swanson, 1988).

It should be remembered that in the present experiment we have only examined experimental changes generated within a single cortical area. As noted earlier, changes must be driven by—and must contribute to—this dramatically emergent behavior within many cortical fields and within several behavioral domains. It is not surprising, then, that nursing behavior can be perturbed by selective lesions that do not include the SI cortex (Beach, 1937). A detailed behavioral assessment beyond that commonly applied in studies of the effects of cortical lesions on nursing behavior, combined with highly selective field-specific lesions—for example, destroying the ventrum representation of SI—will be required to determine how different cortical areas that are remodeled by this behavior contribute to different facets of it.

Several other studies indicate that some aspects of maternal behavior might be learned, and might therefore involve changes in forebrain representations. Thus,

for example, the speed of retrieval of pups is greatly decreased with experience (Beach and Jaynes, 1956; Stern and Kolunie, 1989), the latency before milk injection decreases at longer postpartum times (Jans and Woodside, 1987), and maternal behavior is remembered by an experienced female (Bridges, 1975; Fleming et al., 1990). Indeed, long-term retention of maternal responsiveness requires somatosensory contact with pups via either the snout or ventrum (Morgan et al., 1992). Possible contributions of altered cortical representations to these and to many other postpartum phenomena remain to be determined.

### Some Functional Implications: General Conclusion

These observations are consistent with the conclusion that cortical representations of the skin surfaces are remodeled by our experiences throughout life. By this view, any epochal change in behavior results in changes in representational constructs that account for aspects of its evolution. In the case of nursing, powerful hormonal and reflexive changes drive physical changes that enable and encourage the initiation of suckling. Once suckling is begun, the heavy schedule of novel afferent inputs that it generates produces representational changes that presumably alter the sensations and perceptions generated by suckling. These experience-driven neurological changes become an intrinsic part of the behavior, contributing to its character, maintenance, and maturation.

It should be again emphasized that changes induced in the primary somatosensory (SI) representation reconstructed in these experiments constitute only a small piece of the forebrain changes that are doubtless induced by this behavior. In other experiments in behaviorally trained monkeys, we have recorded experience-induced changes in every mapped somatosensory and motor representational area (Merzenich et al., 1990b). Indeed, alteration of the true primary somatosensory field, area 3b, can be modest in comparison with representational changes generated in other somatosensory representations (see Merzenich, 1990b; Recanzone et al., 1992b). It is probable that human lactation, for example, results in substantial representational remodeling in most or all of more than 10 different somatosensory representational areas, as well as in a number of motor and premotor zones. Moreover, if inputs from the nipples and areolae are segregated from the representation of the surrounding hairy skin in primates as in rats, a second set of forebrain areas are likely to be substantially altered in their representation of this behaviorally important skin, on at least an equivalent scale.

This study constitutes only the beginnings, then, of an effort to reconstruct the complex, distributed, changes that evolve with maternal behavior. Their

elucidation should deepen our understanding of the neurological bases and the ontogeny of this complex epochal behavior. A similar adaptive neurology underlies the genesis and maturation of all new behaviors, which are similarly subject to reconstructive elucidation.

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# IV

## DIMORPHISMS AND COGNITION

As humans, we have a panoply of cognitive behaviors that we seem to think differ between the female and male of the species and that have been the focus of much research over the past 50 years. This section and the proceeding one present chapters reporting on those cognitive differences that have been most studied:

1. Intellectual and spatial abilities;
2. Language processing; and
3. Hemispheric specialization.

Many of these studies are old, but they present the neurobiological evidence (and lack thereof) that underlie the assumption that women are not as skilled at spatial tasks, mathematics, and abstract thinking as men and that men are not as skilled at language and communication as women. Most of the chapters contain at least five short papers investigating these differences in humans. They are short because the experiments are very difficult to carry out and, therefore, are less detailed studies. They are also provocative and so are published in journals such as *Science* and *Nature*, which look for short accounts of studies with wide appeal. Numerous papers are included in each chapter reflecting the fact that each one presents a different outcome; only by looking over the breadth of papers can one begin to see that there really is no consensus—just a vague feeling that women and men think differently being supported at best, by very small differences in brain structures. Two elegant papers use rodent models to study spatial localization showing that it depends on the ability to respond to estrogens via ER beta and the ability to respond to androgens by the presence of the androgen receptor. The applicability of rodent studies to humans remains open to question.

In humans, of course, the opportunity to deprive and supplement with estrogens, as well as breed for the absence of various steroid receptors, would be unethical. Thus, many of the studies take advantage of the variations in circulating hormone action that results from androgen insensitivity and congenital adrenal hyperplasia. It is important to scour these papers for assumptions about human male and female behavior

that we once attributed to sex differences but in current times might be attributed to cultural conditioning. Ask yourself whether, as studies have moved from observing or testing behavior to imaging brain regions under certain behavioral conditions, the differences between female and male have gotten smaller. Also ask yourself whether we have landed on the right paradigms to test. Try to think of paradigms that you think might better test whether or not there are intellectual differences between women and men.

The papers in this section will provide insight into

1. The basic studies that established the notion of cognitive differences in humans;
2. How sex differences in humans have been linked with the organizational/activational paradigm for rodents; and
3. Evidence from animal studies that point to some mechanisms.

Hopefully they will also raise the question, When does a difference make a difference?

### Intellectual and Spatial Abilities

Within Euro-American cultures it has long been assumed that intellectual and spatial abilities of women differ from those of men. Some would say that the impetus to study these differences arose from prejudices about female competence in the world that have long been held across cultures; however, if we were able to study difference without normative claims or claims that one form is better than the other, we would most likely find understanding difference to be enlightening and not encumbering.

The selections in this section are organized to suggest that some differences are useful to understand and others, probably, trivial. It is up to you to decide what to believe based on the science of each study.

Goldman and colleagues' report on the difference between female and male Rhesus in recovery after cortical lesion is a study that, at its heart, is about differences in developmental history that affect the plasticity of prefrontal cortex. While the difference in recovery

from lesion is only present up to approximately one year of age, Goldman suggests that:

A sex-dependent difference in the development of cortical regions could have major implications for the organization of behavior during the formative years and quite possibly for behavioral differences that outlast those years. If so, developmental schedules could be as significant a parameter of sexual dimorphism as structural differentiation itself. (542)

This is important food for thought with respect to data suggesting that women recover language more successfully than men after stroke. It also opens the larger question of what mechanism underlies this developmental difference in plasticity and could the differential timing of steroid hormone secretion in males and females play a role in maintaining plasticity?

Witelson and colleagues' results echo Goldman's in that they find bilateral representation of spatial processing in girls until the age of thirteen, whereas, in boys, bilateral spatial processing ends by approximately age six, once again suggesting that plasticity is maintained longer in the female brain than in the male. Whereas boys are reported to perform more accurately on a spatial task, what is shown is that girls perform slightly less well for each hemisphere but better overall than males processing with only their right hemisphere. One begins to wonder whether the difference in accuracy between the male's and female's right hemisphere performance simply represents different strategies. Furthermore, why is a *lack* of plasticity considered to be a positive influence on spatial processing?

Inglis and Lawson carry this theme further by re-evaluating previous studies of language and intellectual compromise after brain damage. Their innovation is to disaggregate the data by sex. They learn that male patients with left-side lesions show a significant deficit in verbal scale intelligence quotient and women with left-side lesions do not. They conclude that lateralized lesions have very different effects on the intelligence scores of women and men. That this may be the case is supported by both previous and subsequent studies; however, it raises the question again of why greater lateralization and less plasticity has been treated as a standard against which to judge greater or lesser intellectual abilities.

Geschwind and Behan weigh in with their hypothesis that left-handedness, which is more common in males than females, correlates with immune conditions, migraine, and developmental learning disorders located on the behavioral spectrum that defines autism. They also equate mathematical ability with this spectrum and conclude that developmental requisites lead to this constellation of neurological achievements and deficits. This paper is impressive for its wide-ranging thinking around the relationship of handedness, brain

development, steroid hormones, and the immune system. Although we know now that some of the conditions the authors lay at the feet of testosterone delaying development actually occur more commonly in females than in males, it is still instructive to read this example of a deeply thoughtful mind trying to put it all together.

One way of separating the effects of being XY and having androgens is to study androgen-insensitive subjects (AIS). Imperato-McGinley and colleagues use this model in their report on the comparative cognitive abilities of men, women, and AIS study participants from the same family. They control well for socioeconomic status and age but note discrepancies in years of education: AIS = 7.6 years (female, 10.6 years; male, 11 years). Could this play a role in the consistently lower performance of AIS individuals? Interestingly, the study notes that there were left-handed AIS subjects, bringing into question Geschwind and Behan's hypothesis that androgens play a role in the development of handedness. As is clear from previous readings, however, androgens can be converted to estrogens in the brain. In the AIS individuals, unbound circulating androgens could be aromatized to estrogens in the brain, adding a bit of a twist to the androgen story of Geschwind and Behan. Perhaps in some of their behaviors they were actually observing the effects of estrogens?

Finally, Gur and colleagues bring us into the contemporary period of studying sex differences by employing in vivo imaging methods. They use positron-emission tomography to study potential differences in blood flow (read, activation) when female and male subjects are just sitting, independent of task. They find that there may well be different brain activity during resting states with females showing more activity in cingulate cortical regions and men showing more activity in temporal-limbic regions and cerebellum. Their interpretation is that there is a biological substrate for the observation that women are more emotionally adept than men and that the intellectual systems of males are active, even in a resting state.

Read these papers for the variety of approaches and methods they bring to bear on studying age-old assumptions about female and male behavior. Animal models, surveys of humans, disaggregation of data by sex, and in vivo imaging are all brought to bear on intellectual and cognitive differences. This is the modern age of research put to the service of supporting or refuting assumptions based on simple observation. Geschwind and Behan's is the most synthetic, but Goldman's and Witelson's studies make one feel as if they may reveal something biologically fundamental about differences between XX and XY.

Ask yourself which papers contribute the most to your thinking about sex differences in cognition.

### Language Abilities

The idea that females have stronger verbal capabilities than males is also long-standing. It is linked to the arguments around differences in spatial processing since both language and spatial processing are thought to be lateralized or mediated by only one hemisphere. Sex differences in language function have been located in the hypothesis that women have language areas represented bilaterally and males, unilaterally and primarily in the left hemisphere. Interestingly, even the articles purporting to support this difference make us wonder if there is not something less to it than has been promulgated in the popular imagination.

Doreen Kimura's contribution tests the hypothesis that sex differences in the representation of language areas are related to lateralization. Her study uses individuals who have had right or left hemisphere damage due to stroke. It is interesting to note that her analysis reveals that there is, on average, a difference in language areas but that it is not due to unilateral or bilateral representation. Rather, she finds that women recover language better than men after stroke because women have their language areas represented more *anteriorly* in the left hemisphere and, because many strokes occur to vessels located *posteriorly*, men are more affected.

Shaywitz and colleagues' selection has been interpreted as using functional magnetic resonance imaging (fMRI) to demonstrate that women do, indeed, have a more bilateral representation of language areas than men. It is important, however, to note that they report bilateral activation of women's brains only during one language task having to do with phonological processing. Is this the exception that proves the rule? What these findings might encourage would be collaboration between linguists and neuroscientists to devise very sensitive language tasks to enable a detailed study of the aspects of language that are and are not bilaterally represented.

Rossell and colleagues take this admonition seriously and test laterality by monitoring a lateralized lexical visual field task with fMRI. With this task, they find strong left brain activation in males and left and right brain activation in females with females being marginally faster in their response time. Rossell and colleagues note that the clear results of their study depend on the use of a behavioral task known to show a sex difference and admonish other researchers to think through their tasks carefully to avoid the equivocal results. Again, what is found is a sex difference in a very particular language task.

Read these papers for the way in which they review the literature and elucidate the equivocal nature of that literature. Note that there are sex differences but they are not always what has been postulated. One might ask whether there are language tasks that require more bilateral processing, which would in fact yield activation of both hemispheres in men. It is worthwhile asking oneself why bilateral representation would reduce reaction time and accuracy and if it really does to a significant extent. Furthermore, based on the evidence around functional recovery, one might view laterality as a disadvantage because males suffer more severe deficits than females after damage to the left hemisphere due to stroke.

### Hemispheric Specialization

Underlying all of these sex differences in cognition is the idea that the two hemispheres are specialized for different intellectual tasks—left brain/right brain types of tasks. The assumption is that in our behaviors, we may favor one or the other hemisphere. Also embedded in these sex differences is the assumption that lateralization—or using just one hemisphere—is the most successful strategy for speed and accuracy of response. Thus, it is worthwhile looking into the literature that purports to show differences in lateralization between males and females.

Most regions of the brain are, in fact, represented bilaterally via axon bundles that carry information from a given region on one side to its homologue on the other side. These fiber bundles are known as “commissures,” with the major ones being the corpus callosum and the anterior commissure. To support differences in laterality, scientists have long looked for differences in the size, density, and shape of these commissures, attributing more bilaterality to those who have larger commissures and less to those with smaller ones.

Note that the literature shows a tremendous amount of back and forth over whether the corpus callosum is larger in females than in males. One investigator purports to show it is and the next purports to show that it is not. Newer and different methods do not get us any closer to a consensus about any real difference in this structure. Most probably those of us interested in sex differences either have our own, personal favorite paper showing a difference or have come to an uneasy peace consisting of suspicions that there is a difference between female and male but they have yet to be described definitively.

DeLacoste-Utamsing and Halloway present evidence that, in samples from autopsy, there is a difference in the shape and surface area between human female and male corpus callosa with the female being larger.

Oppenheim and colleagues demonstrate, using MRI of living human beings, that there is not.

Halloway and colleagues take a histological approach and show that in their samples, especially those from Australia, there is a difference.

Allen and Gorski tackle both the anterior commissure and the corpus callosum, showing that there is a sex difference in size of the anterior commissure but only one of shape for the corpus callosum in the living human being.

In transgenic mice, Rissman and colleagues show that, at least in females, spatial learning is mediated by estrogen via ER beta—a mechanism that is relatively independent of any of the commissures. This might suggest another hypothesis for mechanisms for behavioral differences.

Jones and Watson take advantage of the naturally occurring androgen insensitive mouse to study androgen's effect on spatial behavior. They find that the behavior of the androgen-insensitive mouse is between that of the male and the female leading them to suggest that the androgen receptor plays a role in the development of male-typical spatial behavior.

Read these papers for the variety of methods they employ and the longevity of the idea that lateralization/bilateralization is a useful mechanism for producing behavior. Also ask yourself whether measuring the external diameter of a group of axons provides enough resolution upon which to base explanations for behavioral differences. Finally, are there really behavioral differences to be interrogated? Would you pursue this sex difference, and, if so, what methods would you use to either prove or disprove this question about female and male minds?

The literature of comparative and physiological psychology contains abundant evidence of behavioral differences between the sexes, not only with regard to the more obvious reproductive activities of the species, but in such nonreproductive behaviors as food preferences, emotionality, aggression, and play (1, 2). Since differences between the sexes in behaviors such as aggression and play may be construed as intimately bound up with reproductive activities, it is not surprising that these, as well as the more plainly evident sexual behaviors, can be modified by gonadal hormones at critical periods in development (1). Further, since gonadal hormones are thought to affect behavior by influencing brain mechanisms (3), it would also not be surprising if neural structures subserving sex-typical behaviors were to differ in males and females. Indeed, anatomical differences between male and female rats have recently come to light in studies of the preoptic area, a region of the brain that has been implicated in reproductive function (4).

In addition to evidence for differences between males and females in reproductive and related activities, substantial evidence exists from studies of human aptitudes for sex differences in performance on intelligence tests (5). However, it is not known to what degree these differences are rooted in cultural patterns and to what degree, if any, they can be attributed to genetically determined dimorphism in neural structures subserving cognitive functions. We have discovered a sex difference in the learning performance of monkeys whose orbital prefrontal cortex had been removed in infancy. Our findings suggest that regions of the neocortex may be sexually dimorphic in nonhuman primates at certain stages of development.

The evidence emerged unexpectedly in the course of experiments concerned with the effects of early cortical and subcortical lesions on cognitive behavior (6). The monkeys of relevance to the present report are those that (i) had been given bilateral orbital prefrontal lesions in infancy (1 to 8 weeks) or as juveniles (18 to 24 months) or were age-equivalent unoperated controls; and (ii) had been tested at various ages on an object discrimination reversal task or on spatial

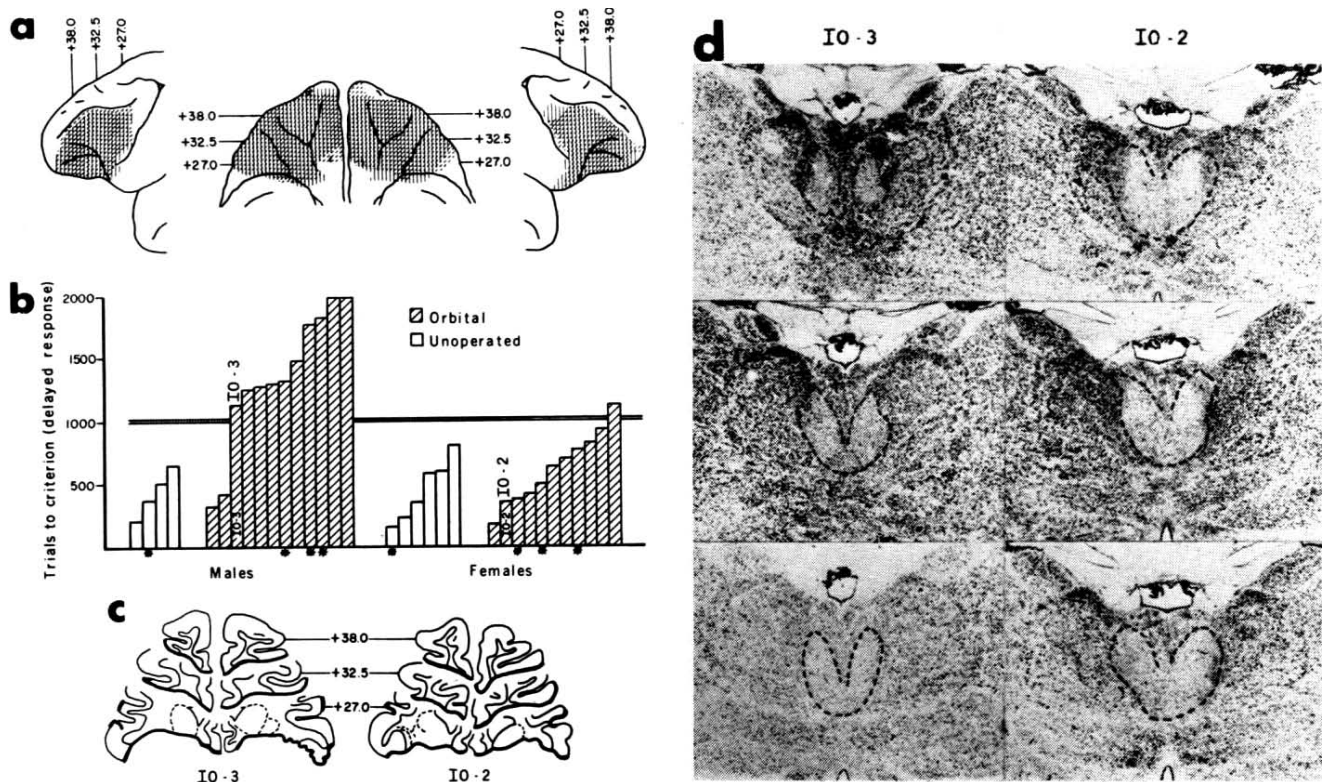
delayed-response problems, tests that are interchangeable as measures of the integrity of orbital prefrontal functions (7).

In all cases, bilateral lesions were made in one stage under aseptic conditions. Infants were anesthetized with ether or methoxyflurane; juveniles, with sodium pentobarbital (40 mg per kilogram of body weight). The ablation involved removal of cortex on the inferior convexity of the frontal lobe and all of the cortical tissue on the ventral surface of the lobe rostral to the Sylvian fissure and lateral to the olfactory stria (figure 57.1a).

The object reversal test involved training monkeys to discriminate between two objects differing in color, size, and shape. After the animals reached criterion (two successive 30-trial sessions with 90 percent correct in each session), the reward contingencies were reversed so that the previously positive object became negative. The monkey's score for this task was the total number of errors to criterion made over six reversals. In the delayed-response task, the monkey was trained to observe the experimenter conceal a bait in the left or right of two food wells located on a test board in front of the animal. The position of the baited well on successive trials was governed by a modified random order. On any given trial, the monkey could select the baited food well only after an opaque screen had been interposed between the monkey and the test board for up to 5 seconds. In a related task, delayed alternation, the monkey was required to alternate between the left and right food wells on successive trials separated by 5-second intervals. The monkey's score for each of these tasks was the number of trials required to achieve a performance criterion of 90 correct responses in 100 consecutive trials. Detailed procedures for behavioral testing have been described (6).

When testing was completed, the brains of the operated monkeys were perfused and processed for histological and anatomical analysis. To date, anatomical results are available for 21 of the operated cases. The remaining monkeys are still being studied.

The first study involved eight early operated and nine unoperated monkeys of both sexes that were



**Figure 57.1**

(a) Examples of two lesions reconstructed on standard ventral and lateral views of the monkey brain. Interrupted lines denote the lesion of IO-2; uninterrupted lines, lesion of IO-3. (b) Performance of 12-month-old monkeys on delayed response. Asterisks indicate monkeys that had previously been tested on object reversal at 2½ months of age. IO-2 and IO-3 are the monkeys whose anatomical data are presented in (a), (c), and (d). (c) Cross sections through the brains of IO-2 and IO-3. Lesions are indicated by heavy lines. (d) Photomicrographs of thalamic degeneration in the brains of IO-2 and IO-3 at 0.5-mm intervals through the anterior portion of the dorsomedial nucleus. The boundaries of the degeneration have been marked by interrupted lines ( $\times 13$ ).

tested at 2½ months of age (object reversal). The results (table 57.1) showed that the operated males were impaired relative to unoperated males ( $P < .032$ ), while operated females performed as well as unoperated females.

In a second study, 33 monkeys that had been operated in infancy were tested at 12 months of age (delayed response). Eight of these animals had been tested on object reversal at 2½ months of age. Again, the results were sex-dependent: the operated males were impaired compared both to their controls ( $P < .02$ ) and to the operated females ( $P < .02$ ), which in turn did not differ from unoperated females (figure 57.1b). Also, the results were not influenced by prior testing experience. The difference between operated males and operated females was obtained in the monkeys that had been tested previously as well as in those that had not (figure 57.1b).

Examination of the brains by light microscopic methods has so far failed to provide evidence of more pathology in males than in females either with regard to the extent of the lesions or the retrograde degeneration in the dorsomedial nucleus of the thalamus. Thus,

the different effects of orbital prefrontal lesions in males and females are probably not due to differences in the size of the lesions. Figure 57.1, a, c, and d, presents, respectively, reconstructions of the lesions, serial sections through these lesions, and serial sections through the thalamus of a male and female that were tested on delayed response at 12 months of age. The cortical lesions of the two animals were similar, but the retrograde changes in the dorsomedial nucleus were more extensive in the female than in the male. In spite of this, the male and not the female was impaired on delayed response (figure 57.1b).

It is unlikely that this sex-dependent effect of neocortical lesions reflects a permanent difference in the functions of the orbital prefrontal cortex in males and females. All of the monkeys tested at 12 months of age on one of the measures of orbital function (delayed response) were subsequently tested at approximately 15 and 18 months of age on the other measures (delayed alternation and object reversal, respectively). At 15 months of age, more than half of the operated females began to exhibit deficits as severe as those displayed by operated males, and the two groups were not signifi-

**Table 57.1**  
Summary of behavioral results

Age at Testing (Months)	Test	Unoperated			Operated		
		Mean	Range	<i>N</i>	Mean	Range	<i>N</i>
<i>Males</i>							
2½	Object reversal	168	120–236	5	277	192–360	4
12	Delayed response	430	200–640	4	1334	310–2000	12
15	Delayed alternation	570	120–950	4	1487	450–2000	12
18	Object reversal	97	57–132	4	139	71–221	12
24+	Object reversal	84	45–120	3	337	263–458	3
24+	Delayed response	420	300–540	4	825	470–1240	4
27+	Delayed alternation	367.5	50–560	4	1895	1160–2000	8
<i>Females</i>							
2½	Object reversal	219	163–244	4	202	131–261	4
12	Delayed response	455	150–810	6	619	360–1140	11
15	Delayed alternation	682	60–1380	6	1178	200–2000	11
18	Object reversal	91	54–145	6	131	82–221	11
24+	Object reversal	68	51–91	5	161	89–301	4
24+	Delayed response	425	120–760	4	1098	980–1220	4
27+	Delayed alternation	292.5	10–530	4	2000	2000–2000	4

Scores are given as errors to criterion for object reversal and as trials to criterion for delayed alternation; *N*, number of animals. A one-tailed Mann-Whitney U test was used to evaluate differences between operated groups and controls of the same sex; all other comparisons were two-tailed. There were no significant differences between unoperated groups at any age. Operated males were significantly impaired ( $P < .05$ ) relative to unoperated males at all ages, whereas operated females were not impaired ( $P < .05$ ) relative to unoperated females (and unoperated males) until 18 months of age or later. In addition, operated males performed significantly worse than operated females at 12 months of age ( $U = 20$ ,  $P < .02$ ). Estimates of absolute performance levels at different chronological ages are valid only for comparisons between independent groups given comparable test experience at different ages. Animals tested on object reversal at 2½ months versus 24 months or later; animals tested on delayed response at 12 months versus 24 months or later; and those tested on delayed alternation at 15 months versus 27 months or later.

cantly different (table 57.1). Nevertheless, it was still the case that only the operated males differed significantly from their unoperated controls ( $P < .05$ ). By 18 months of age, however, the operated females not only performed as poorly as the operated males, but both groups were impaired similarly relative to controls ( $P < .05$  in both cases). The results of these longitudinal investigations are consistent with those of two further studies involving independent groups of older monkeys. In one study, monkeys given prefrontal lesions as juveniles and unoperated age-equivalent controls were first tested on object reversal at approximately 24 months of age. In another study, monkeys given orbital prefrontal lesions either in infancy or as juveniles were also first tested at 24 months of age or older but on delayed response. Animals from both studies were then trained on delayed alternation when they were nearly 27 months of age at the youngest. No significant sex differences emerged on any of these measures (table 57.1). Further, both operated females and operated males were markedly impaired relative to controls ( $P < .01$  in all instances where statistical tests were necessary). The negative findings in older monkeys are in accord with the lack of evidence in the literature for sex differences in the learning abilities of brain-damaged monkeys.

The present results suggest that, up to about 1 year of age, orbital prefrontal lesions result in impairments

on object reversal or delayed response in males compared to females, whereas at later ages, 15 to 18 months and beyond, the lesions induce deficits in both sexes to the same degree. The variety of conditions under which behavioral determinations have been made indicate that neither the age of the animal at surgery, the particular test given, nor interactions between tests are critical factors in these results. Rather, what is important is the sex of the animal and the age at testing. In spite of the similar performance of unoperated groups, the finding that lesion-induced deficits can be detected at earlier ages in males than in females may be regarded as evidence that the functions of the orbital cortex develop earlier in males than in females (8). Previous conclusions (6) with regard to the functional status of the orbital prefrontal cortex at different stages of ontogenetic development should now be qualified in relation to gender.

A sex-dependent difference in the development of cortical regions could have major implications for the organization of behavior during the formative years and quite possibly for behavioral differences that outlast those years. If so, developmental schedules could be as significant a parameter of sexual dimorphism as structural differentiation itself. Developmental differences in neural maturation may conceivably have a bearing also on many central nervous system disorders, such as cerebral palsy or specific developmental

dyslexia, which appear to have a higher incidence in boys than in girls (9). To the extent that perinatal injuries are implicated in some forms of these disorders, such injuries may exact consequences with higher probabilities in males than in females because of regional differences in brain growth at the time of insult. Finally, the present findings raise the possibility that developmental disparities between the sexes will be found for other brain regions as well, although the direction of the differences may vary with the behavioral functions involved. However, it is also possible that only structures like the orbital region become functionally mature according to different timetables in males and females because of especially intimate connections with the limbic system and hypothalamus.

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4. G. Raisman and P. M. Field, *Brain Res.* 54, 1 (1973).
5. E. E. Maccoby, in *The Development of Sex Differences*, E. E. Maccoby, Ed. (Stanford Univ. Press, Stanford, Calif., 1966), pp. 25–55; L. F. Jarvik, paper presented at a conference on Perspectives on the Nature of Intelligence, Mental Retardation Center, University of California, Los Angeles, 1971.
6. P. S. Goldman, *Exp. Neurol.* 32, 366 (1971); E. A. Miller, P. S. Goldman, H. E. Rosvold, *Science* 182, 304 (1973); P. S. Goldman, in *CNS Plasticity and Recovery of Function*, D. Stein, J. Rosen, N. Butters, Eds. (Academic Press, New York, in press); ———, H. E. Rosvold, M. Mishkin, *J. Comp. Physiol. Psychol.* 70, 454 (1970).
7. C. A. Butter, *Physiol. Behav.* 4, 163 (1969); P. S. Goldman, *Exp. Neurol.* 32, 366 (1971).
8. Although there was no evidence in the present study of a difference in performance of unoperated males and females at any stage of development, there have been a few reports in intact juvenile and adult rhesus monkeys of female superiority on delayed responses [A. J. Blomquist, thesis, University of Wisconsin (1960), No. 60-5717, University Microfilms (Ann Arbor, Mich., 1960); A. A. McDowell, W. L. Brown, A. C. McTee, *J. Comp. Physiol. Psychol.* 53, 429 (1960)]. Such findings leave open the possibility that there are more permanent differences between the sexes in the neural mechanisms underlying delayed response than the present study indicates, or that slower development of the orbital cortex ultimately confers a functional advantage on females.
9. A. Towbin, *The Pathology of Cerebral Palsy* (Thomas, Springfield, Ill., 1960), p. 90; M. Critchley, *The Dyslexic Child* (Thomas, Springfield, Ill., 1970), pp. 90–92.

The right hemisphere plays the major role in adults in nonlinguistic, spatial, and holistic cognitive processing, which includes two (possibly related) sets of skills: (i) visual- and tactile-spatial processing and (ii) nonlinguistic and nonsequential auditory processing (1). Study of hemisphere specialization during development has focused on the left hemisphere (2–6) and relatively ignored the right (7–9), particularly its role in spatial processing. Unlike language, spatial perception is prominent in the behavior of both human infants and nonhuman species. Consequently, hemisphere specialization for spatial processing may be critical in human ontogenetic and possibly in phylogenetic development of lateralization of function in general, and it is an important aspect of the neural substrate of cognition.

I now report some initial information concerning the early course of specialization of the right hemisphere for spatial processing, with regard to both age and sex. In the course of experiments with children, it was discovered that, in boys, the right hemisphere has the dominant role in processing nonlinguistic spatial information by at least age 6 years; in contrast, in girls the right hemisphere is not dominant even by age 13 years, but rather, there is bilateral representation. These results suggest (i) that boys have greater hemisphere specialization and (ii) that there is a sexual dimorphism in the neural organization related to cognition for an extended period of development.

Suitable methods for studying specialization of the right hemisphere in children have been lacking (10). I used a new behavioral test procedure involving tactual perception, which was devised specifically to assess the relative participation of the two hemispheres in spatial processing in neurologically intact children (11). In essence, the test requires that the subject palpate simultaneously, out of view, two different meaningless shapes for 10 seconds, each one with the index and middle fingers of one hand. He then tries to choose these two shapes from a visual display containing six such shapes. After many practice trials, ten test trials are given; the scores are the number of left- and right-hand objects correctly chosen. The test has two crucial

features. (i) It requires tactile shape discrimination, which, in adults, depends mainly on the right hemisphere (12). Furthermore, to make the test as dependent on the right hemisphere as possible, the stimuli were designed to be meaningless shapes, not readily labeled; the simultaneous palpation of different stimuli tends to hinder linguistic encoding; and the incorrect items in the recognition display were designed to have details similar to those of the test stimuli, so that a correct response depends on a gestalt perception of the whole stimulus. (ii) Different stimuli are presented simultaneously, here termed “dichhaptic” stimulation: “dich,” from dichotomy, to refer to the simultaneous and different stimulation; and “haptic,” referring to active touch. It was hoped that this procedure would produce competition in the neural system (13, 14) such that any superiority of the right hemisphere for the required cognitive processing would be reflected in superior perception of the contralateral (left) hand stimuli (15). Such was the empirical result in an earlier study of a small group of boys (11), but the present study indicates it to be so only for boys.

I studied 200 right-handed children, as defined by consistent preference for the right hand for writing and on at least eight of ten unimanual tasks. There were 25 subjects of each sex within each 2-year interval from 6 to 13 years, all with at least normal tested intelligence, age-appropriate academic achievement, and normal medical and behavioral status. Each child was given the dichhaptic stimulation test. The boys, but not the girls, obtained greater left- than right-hand scores (figure 58.1). A mixed design analysis of variance was done with sex and age as the between-subject variables and hand as the within-subject variable. There was no difference in overall accuracy between boys and girls ( $F < 1.0$ ); overall performance improved with age in a linear fashion [ $F(3, 192) = 24.2$ ,  $P < .0001$ ]; hand was a significant factor [ $F(1, 192) = 7.1$ ,  $P < .01$ ]. In addition, there was a significant interaction between hand and sex [ $F(1, 192) = 6.8$ ,  $P < .01$ ]. No other interactions were significant ( $F < 1$  in all cases). The left-hand score of the boys (5.4) was significantly

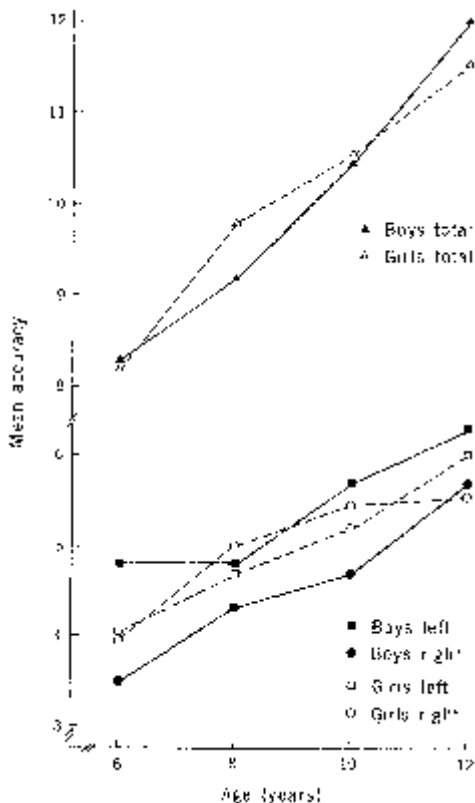


Figure 58.1

Mean accuracy scores for recognition of nonsense shapes presented to the left and right hands on a dichaptic stimulation test. Maximum possible score per hand is 10.

better than their right-hand score (4.6). There was no difference between hands (5.0 and 5.0) for the girls. The difference between the sexes for each hand was not significant ( $.05 < P < .10$ , in both cases). The Duncan Multiple Range Test (16) was used for all comparisons. Significantly more boys than girls had better left- than right-hand scores ( $\chi^2 = 5.28$ , d.f. = 1,  $P < .05$ ). Since one of the a priori questions concerned the age at which evidence for asymmetry is first observed, I tested to determine whether the difference between hand scores was significant for the youngest subgroup of boys, and it was ( $t = 3.20$ , d.f. = 24,  $P < .01$ ).

Each child was also given a typical verbal dichotic listening test as an index of left hemisphere specialization for language (17). Both boys and girls showed greater accuracy for right- than left-ear stimuli ( $t = 5.09$ , d.f. = 86,  $P < .001$ ;  $t = 2.76$ , d.f. = 88,  $P < .01$ , respectively) (18). A chi-square analysis of the number of girls and boys with greater right- or left-ear scores indicated no difference between the sexes ( $\chi^2 = 0.03$ , d.f. = 1,  $P > .85$ ).

The greater left- than right-hand score for the boys is consistent with earlier results (11). The difference in

hand asymmetry between the sexes cannot be attributed to differential asymmetry in tactile sensitivity (19). Nor is it likely that the sex difference is due to greater use by girls than boys of a left hemisphere verbal-analytic strategy, as the test was devised to make the use of such strategies unlikely (if not impossible) and useless. Finally, the laterality difference between the sexes was specific to the spatial task. In both groups, the right ear was superior on the verbal dichotic test.

I have argued (11) that superiority of the left hand on this dichaptic test reflects superiority of the right hemisphere for spatial processing. Within this context, I now suggest that for boys of at least 6 the right hemisphere is more specialized than the left for spatial processing; in girls, however, there is bilateral representation at least until adolescence. Thus, the same neural structures in males and females may have different functions with respect to at least one aspect of cognition during a major period of development. Conversely, the same cognitive process may be mediated by different parts of the brain in boys and girls.

The results are consistent with those of the few reports that studied the sexes separately for lateralization of spatial processing. Several studies with adults (20) and the one study with children (21) suggest greater participation of the left hemisphere in spatial tasks in females than in males. In contrast, no such sex difference has been observed in children for specialization of the right hemisphere for nonlinguistic auditory perception (22) or for specialization of the left hemisphere for language (23).

The most direct evidence for the existence of sexual dimorphism in neural organization underlying cognition during development comes from recent studies with rhesus monkeys (24). Bilateral lesions in infancy of orbital prefrontal cortex resulted in impaired performance on tests involving, interestingly, spatial discrimination in male monkeys when tested in infancy ( $2\frac{1}{2}$  months) and again at maturity; but in females, early lesions did not induce deficits until the animals reached about 15 to 18 months of age.

Sexual dimorphism in the neural organization underlying cognition may have educational implications. Reading is considered to involve both spatial and linguistic processing (25). The brains of girls and boys may be differentially organized for the cognitive processes involved in reading at a time in development when they are learning to read. Therefore, different approaches in teaching reading, such as the "look-say" and phonetic methods, that stress different cognitive strategies and, by inference, depend on different neural structures in the two sexes, may be differentially effective for each sex.

The superiority of males to females on many, although not all, spatial tests (26) may be related to the hypothesized neural dimorphism. Spatial ability seems to be related to sex chromosomes (27) and testosterone levels (28). Such genetic and hormonal factors may cause the neural dimorphism in the sexes (29), which in turn may underlie the sex difference in spatial ability. Interdependence of these physiological, neural, and cognitive factors suggests a relatively unexplored and possibly fruitful dimension for the study of (i) individual differences in cognition; (ii) the etiology of clinical disorders possibly associated with atypical patterns of neural lateralization, particularly of specialization of the right hemisphere (such as developmental dyslexia) (30); and (iii) the potential use of sex hormones in the clinical management of neural trauma and developmental disorders in which an extended period of neural plasticity and hemisphere equipotentiality may be beneficial (31).

If the right hemisphere in girls is not specialized for a particular cognitive function, then the brain of young females, particularly the right hemisphere, may have greater plasticity for a longer period than that of males. Thus, language functions may transfer more readily to the right hemisphere in females than in males following early damage to the left hemisphere; in fact, for patients with early lesions, women show less impairment than men on verbal tasks after neurosurgical removal of left hemisphere tissue at maturity (32).

Greater plasticity of the young female brain also suggests that females may have a lower incidence of developmental disorders associated with possible left hemisphere dysfunction and for which greater plasticity of the right hemisphere might be advantageous. Males do have a higher incidence than females of developmental dyslexia (33), developmental aphasia (34), and infantile autism (35), all of which have language deficits as a predominant symptom.

## References and Notes

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2. Evidence for early left hemisphere specialization for language comes from several diverse sources: (i) studies of language deficits in brain-damaged children [for example, L. S. Basser, *Brain* 85, 427 (1962); M. Annett, *Cortex* 9, 4 (1973)]; (ii) studies with normal children using verbal dichotic listening tests [for example (3–5)]; (iii) studies using cortical evoked potentials to speech sounds (6); and (iv) less directly, from reports of neuroanatomical asymmetry in language areas in neonates [for example, S. F. Witelson and W. Pallie, *Brain* 96, 641 (1973); J. A. Wada, R. Clarke, A. Hamm, *Arch. Neurol. (Chicago)* 32, 239 (1975)].
3. D. Kimura, *J. Comp. Physiol. Psychol.* 56, 899 (1963).
4. D. Ingram, *Neuropsychologia* 13, 103 (1975).
5. A. K. Entus, in *Language Development and Neurological Theory*, S. Segalowitz and F. Gruber, Eds. (Academic Press, New York, in press).
6. D. L. Molfese, R. B. Freeman, Jr., D. S. Palermo, *Brain Lang.* 2, 356 (1975).
7. In children, right hemisphere superiority for non-linguistic auditory processing is indicated on the basis of nonlinguistic dichotic tests (5, 8) and electrophysiological recordings (6). Superiority of the right hemisphere for spatial processing is suggested by the superiority of the left hand in reading Braille symbols [B. Hermelin and N. O'Connor, *Neuropsychologia* 9, 431 (1971); Rudel et al. (9)]; and by a few studies with brain-damaged children [for example, R. G. Rudel, H.-L. Teuber, T. E. Twitchell, *Neuropsychologia* 12, 95 (1974)].
8. C. Knox and D. Kimura, *Neuropsychologia* 8, 227 (1970).
9. R. G. Rudel, M. B. Denckla, E. Spalten, *Neurology* 24, 733 (1974).
10. The various possible strategies [such as (i) naturalistic observation of clinical cases; (ii) objective study of cognitive deficits in individuals with unilateral brain damage, hemispherectomy, or commissurotomy; and the study, in normal individuals, of (iii) visual field asymmetry with tachistoscopic stimulation and (iv) electrophysiological asymmetry] have all been, for technical or practical reasons, of limited use in the study of the role of the right hemisphere in spatial processing in early childhood.
11. S. F. Witelson, *Cortex* 10, 3 (1974).
12. For example, R. D. Nebes, *ibid.* 7, 333 (1971); *Brain* 95, 633 (1972); B. Milner and L. Taylor, *Neuropsychologia* 10, 1 (1972).
13. Simultaneous but different stimulation to the two ears (dichotic listening) is a useful tool in tapping hemisphere functional differences (14). Analogously, dichaptic stimulation was devised for the tactual modality. When one tactile object is presented to either hand, there is no difference between the hands in accuracy for shape recognition [for example, A. Carmon and A. L. Benton, *Neurology* 19, 525 (1969); E. De Renzi and G. Scotti, *Cortex* 5, 53 (1969)]. However, for some tactile-spatial tasks, such as the perception of direction of brief tactile stimulation to the hands, even unimanual stimulation may yield perceptual asymmetry [A. L. Benton, H. S. Levin, N. R. Varney, *Neurology* 23, 1248 (1973)].
14. D. Kimura, *Cortex* 3, 163 (1967).
15. Ipsilateral somesthetic pathways contribute only to gross perception, such as the presence or absence of tactile stimulation [R. W. Sperry, M. S. Gazzaniga, J. E. Bogen, in *Handbook of Clinical Neurology*, P. J. Vinken and G. W. Bruyn, Eds. (North-Holland, Amsterdam, 1969), vol. 4, pp. 273–290].
16. D. B. Duncan, *Biometrics* 11, 1 (1955).
17. The particular test used involved free recall of series of three dichotic pairs of digits, presented at the rate of two pairs per second. On such tests, accuracy is greater for material presented to the right than to the left ear in adults [for example (14)] and in children [for example (3, 4)], which is considered to reflect left hemisphere specialization for linguistic processing.
18. The total number of subjects in each sex group for this test is less than 100 due to the exclusion of those subjects whose audiometric testing indicated depressed or differential ear acuity.
19. Although some studies with adult men and women [for example, S. Weinstein, in *The Skin Senses*, D. R. Kenshalo, Ed. (Thomas, Springfield, Ill., 1968), pp. 195–222] indicate greater pressure sensitivity for the left fingers and palm, whereas other studies report no laterality difference [for example, A. Carmon, D. E. Bilstrom, A. L. Benton, *Cortex* 5, 27 (1969)], the one study with children reports greater left sensitivity in girls by the age of 6, but in boys only by the age of 11 [L. Ghent, *J. Comp. Physiol. Psychol.* 54, 670 (1961)]. In contrast, in my study boys as young as age 6 showed a left-hand superiority for shape discrimination; girls did not, in spite of possibly greater left-finger sensitivity.
20. H. Lansdell, *Nature (London)* 194, 852 (1962); *Cortex* 4, 257 (1968); D. Kimura, *Can. J. Psychol.* 23, 445 (1969); J. E. Bogen, R. DeZure, W. D. TenHouten, J. F. Marsh, *Bull. Los Angeles Neurol.*

- Soc.* 37, 49 (1972); J. McGlone and W. Davidson, *Neuropsychologia* 11, 105 (1973); J. McGlone and A. Kertesz, *Cortex* 9, 313 (1973); J. McGlone, paper presented at the 4th Annual Meeting of the International Neuropsychology Society, Toronto, Ont., 4 to 7 February 1976.
21. The study of Braille reading by Rudel et al. (9) suggests greater participation of the right hemisphere on this task in boys by the age of 11 years but not in girls even by the age of 13. The late appearance of left-hand superiority in boys in this study may be related to the verbal component in the particular task used, and, by inference, to left hemisphere processing. One study using a "conflict drawing test" [A. W. H. Buffery and J. A. Gray, in *Gender Differences: Their Ontogeny and Significance*, C. Ounsted and D. C. Taylor, Eds. (Churchill Livingstone, London, 1972), pp. 128-157] suggests the opposite hypothesis, that boys develop right hemisphere specialization for spatial functioning later than girls. However this interpretation does not follow unequivocally from their data.
22. Although Knox and Kimura (8) found that overall (left plus right) accuracy for recall of environmental sounds was greater for boys than girls, superiority of the left ear occurred as early and was of the same magnitude in girls as in boys. Entus (5) also found no sex differences.
23. No data indicative of sex differences in speech representation are reported in studies of acquired aphasia in children. Many studies using verbal dichotic tests in normal children analyzed performance of the sexes separately, and most found no sex difference in age of onset, incidence, or magnitude of superiority of the right ear [for example, Kimura (3); Knox and Kimura (8); C. I. Berlin, L. F. Hughes, S. S. Lowe-Bell, H. L. Berlin, *Cortex* 9, 393 (1973); Entus (5)]. A few studies did find no superiority of the right ear in some age-sex groups: in some cases for girls, in others for boys, and sometimes at age levels beyond those for which significant ear asymmetry was obtained [for example, Kimura (14); M. Nagafuchi, *Acta Oto-Laryngol.* 69, 409 (1970); Ingram (4)]. These data do not provide evidence of a sex difference in lateralization of language functions in children.
24. P. S. Goldman, H. T. Crawford, L. P. Stokes, T. W. Galkin, H. E. Rosvold, *Science* 186, 540 (1974).
25. E. J. Gibson, *Cognitive Psychol.* 2, 351 (1971).
26. For reviews, see L. M. Terman and L. E. Tyler [in *Manual of Child Psychology*, L. Carmichael, Ed. (Wiley, New York, ed. 2, 1954), pp. 1064-1114] and L. J. Harris [in *Hemispheric Asymmetries of Function*, M. Kinsbourne, Ed. (Cambridge Univ. Press, Cambridge, England, in press)].
27. J. Money, *J. Psychiat. Res.* 2, 233 (1964); R. D. Bock and D. Kolakowski, *Am. J. Hum. Genet.* 25, 1 (1973).
28. J. L. M. Dawson, *Int. J. Psychol.* 2, 171 (1967); D. N. Masica, J. Money, A. A. Ehrhardt, V. G. Lewis, *Johns Hopkins Med. J.* 124, 34 (1969).
29. A somewhat analogous situation is that the role of the ventromedial hypothalamus in response to aversive stimulation in the rat is dependent on the sex of the animal and on early levels of testosterone [M. Dennis, *Exp. Neurol.* 37, 256 (1972); *Physiol. Behav.*, in press].
30. S. F. Witelson, in *The Neuropsychology of Learning Disorders: Theoretical Approaches*, R. Knights and D. J. Bakker, Eds. (University Park Press, Baltimore, in press).
31. F. Nottebohm [*Science* 167, 950 (1970); *J. Exp. Zool.* 179, 35 (1972)] reports that lateralization of neural control for bird song is fixed and plasticity is no longer possible by the end of song learning, and that termination of the critical period for normal full song development is postponed by reduced early testosterone concentrations. Therefore, reduced testosterone may prolong the period of latent neural plasticity.
32. H. Landsdell, *Am. Psychol.* 16, 448 (1961); *J. Abnorm. Psychol.* 81, 255 (1973).
33. For example, M. Critchley, *The Dyslexic Child* (Heinemann, London, 1970), p. 91; Witelson (30).
34. A. L. Benton, *Cortex* 1, 40 (1964); T. T. S. Ingram, *Brain* 82, 450 (1959).
35. M. Rutter, L. Bartak, S. Newman, in *Infantile Autism: Concepts, Characteristics and Treatment*, M. Rutter, Ed. (Churchill Livingstone, London, 1971), pp. 148-171.
36. Supported by the Ontario Mental Health Foundation research grant 322. I thank the staff members of the Wentworth County Roman Catholic Separate School Board for their cooperation in providing subjects; H. Evenden, M. Irvine, J. Swallow, and D. Clews for technical assistance; and A. B. Kristofferson and J. Diamond for their constructive comments on earlier drafts of this report. Some of these data were presented in a paper given at the Biennial Meeting of the Society for Research in Child Development, Denver, Colo., 10 to 13 April 1975.

For some 30 years neuropsychologists have reported contradictory findings concerning deficits in performance on the intelligence tests of patients who have suffered unilateral brain damage. Investigators such as Andersen (1) found that left hemisphere damage significantly reduces scores on verbal tests and right hemisphere damage significantly reduces scores on nonverbal tests; we call this the positive case of the test-specific laterality effect. Other investigators such as Reitan (2) reported a significant effect only in the case of left hemisphere damage on verbal test scores; this is an example of the equivocal case. Still others, such as Smith (3), failed to find any differential effects of lateralized brain damage on either verbal or nonverbal cognitive test performance; this we call the negative case. Some of these apparent contradictions can be resolved by taking the sex of the patients studied into account. This has not been the practice, with the exception of some work by Lansdell (4).

McGlone (5), however, reported that only the male patients in her studies showed a significant lateralized effect of brain damage, those with left hemisphere damage being impaired on the Verbal Scale and those with right hemisphere damage being impaired on the Performance Scale of the Wechsler (6) intelligence test. The female patients did not show selective deficits on these scales after comparable brain damage in one cerebral hemisphere or the other.

In light of McGlone's results, we investigated two possibilities. First, in those cases in which significant verbal and nonverbal deficits have been reported in groups with left and right brain damage, respectively (that is, in the positive case), there should be many more male than female patients. Conversely, in studies with either equivocal or negative outcomes there should be a greater proportion of female patients; because the test results obtained from women with brain damage do not show lateralized effects, they would mask the trends found in men.

Many investigators in this area neglected to describe the sex distribution of their patient groups. Table 59.1 shows the composition of positive studies (1, 7–10) and of equivocal and negative studies (9, 11–18) for cases

in which the sex of the patients was reported. This tabulation bears out our first expectation. With only two exceptions (16, 17), the balance of the sexes is quite different in the two groups, with a much greater ratio of male to female patients in the group of positive cases.

The second possibility we investigated was that the data from studies with equivocal and negative outcomes would show test-specific laterality differences between the male patients with left and right hemisphere damage if test results were reexamined. We carried out such an analysis for cases of left- and right-sided temporal lobectomy reported by Meyer and Jones (11). They had tested patients on the Wechsler-Bellevue scales (19) 1 week before and 1 month after temporal lobectomy and found that only the patients with left-sided temporal lobectomy showed a significant deficit on the Verbal Scale. Although these authors did not

**Table 59.1**

The composition of the patient groups in the positive and equivocal or negative cases of the test-specific laterality effect of brain damage

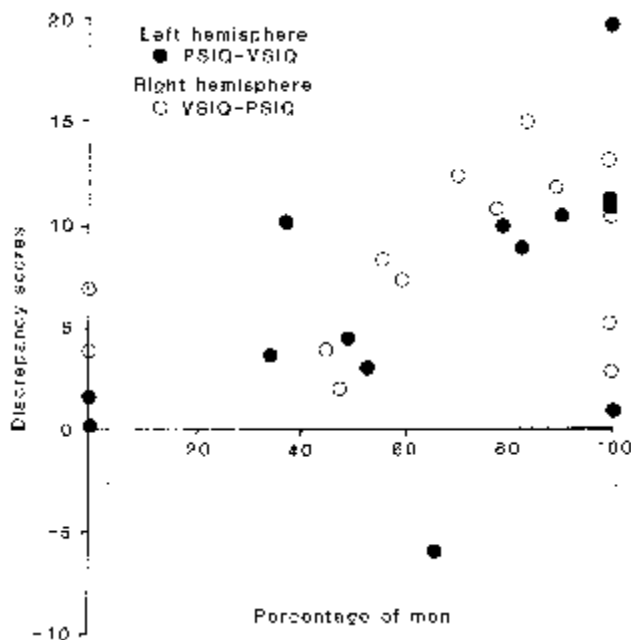
References	Male Patients		Female Patients	
	Left Lesion	Right Lesion	Left Lesion	Right Lesion
<i>Positive Cases</i>				
Andersen (1)	15	15	0	0
Klove and Reitan (7)	19	28	3	8
Klove (8)	33	33	9	4
Fitzhugh, Fitzhugh, Reitan ("current" cases) (9)	15	21	3	4
Fields and Whitmyre (10)	18	23	0	0
Total	100	120	15	16
<i>Equivocal or Negative Cases</i>				
Meyer and Jones (11)	11	5	9	6
Fitzhugh, Fitzhugh, Reitan ("chronic" cases) (9)	7	12	13	13
Klove and Fitzhugh (12)	12	19	12	16
Fitzhugh and Fitzhugh (13)	14	11	14	13
Dennerll (14)	11	22	18	9
Meier and French (15)	8	14	7	11
Zimmerman, Whitmyre, Fields (16)	23	31	0	0
Reitan and Fitzhugh (17)	15	15	0	0
Todd, Coolidge, Satz (18)	45	27	23	19
Total	146	156	96	87

**Table 59.2**

Mean discrepancies between expected and actual postoperative Verbal Scale and Performance Scale IQ's and their associated standard errors (S.E.)

Scale	Male Patients				Female Patients			
	Left Lesion ( <i>N</i> = 11)		Right Lesion ( <i>N</i> = 5)		Left Lesion ( <i>N</i> = 9)		Right Lesion ( <i>N</i> = 6)	
	$\bar{X}$	S.E.	$\bar{X}$	S.E.	$\bar{X}$	S.E.	$\bar{X}$	S.E.
VSIQ	-10.73**	3.20	-3.40 (N.S.)	3.50	-12.00**	3.48	+7.00 (N.S.)	3.02
PSIQ	-4.64 (N.S.)	2.25	-5.80*	2.01	-12.89*	3.90	+2.67 (N.S.)	2.95

Negative scores indicate a deficit; \*\**P* .01; \**P* .05; N.S., not significant. [Data from (11)]

**Figure 59.1**

Scattergram of Verbal Scale IQ and Performance Scale IQ discrepancy scores for cases of left hemisphere brain damage ( $PSIQ - VSIQ$ ) and right hemisphere damage ( $VSIQ - PSIQ$ ).

examine the influence of sex, they specified the sex of each patient as well as the individual test results both before and after surgery. We reanalyzed their data for differences by sex by calculating the expected postoperative IQ scores on form 2 of the Wechsler for each patient, using that patients' preoperative scores from form 1. These calculations are based on the test-retest regression data provided by Gibby (20) and Gerboth (21). The differences between the expected scores and the scores actually obtained by the patients upon retesting were then tabulated, taking into account both the sex of the patient and the laterality of the surgical lesion (table 59.2).

The male patients with left-sided brain lesions showed a statistically significant deficit in Verbal Scale IQ (VSIQ), and right-sided lesions produced a statistically significant Performance Scale IQ (PSIQ) deficit in the men. No such patterns are apparent in the data

from the female patients. Women with left hemisphere damage in this study suffered a statistically significant decline on both the Verbal and the Performance scales, but these postoperative deficits were not test-specific. The women with right-sided brain lesions did not show a statistically significant change on either scale.

Using data from studies in table 59.1 (7-9, 11, 13-18) as well as McGlone's data (5), we plotted the actual values of the differences between VSIQ and PSIQ against the percentage of males in each group with either right or left hemisphere brain damage (figure 59.1). We omitted those studies that did not provide the actual discrepancies between Verbal and Performance scale scores.

This distribution (22) (figure 59.1) represents a rank-order correlation of +0.51 ( $P < .01$ ) between the magnitude of the discrepancy scores and the percentage of men in each group. Such a high degree of correlation is striking in view of the great heterogeneity of the groups of patients under consideration.

Bryden (23) and McGlone (24), who reviewed studies on sex-related differences in cerebral organization, including studies of dichotic listening, tachistoscopic perception, and sensorimotor performance, concluded that sex-related differences in the functional asymmetry of the human brain do exist, but neither reviewed the results of the studies described in this report.

We believe that the studies we examined, upon reanalysis, unequivocally support McGlone (5) insofar as they confirm that lateralized brain lesions produce very different effects on the intelligence test scores of men and women. Our data further demonstrate that such sex differences influenced the results of earlier investigations. These findings indicate the need for the reevaluation of much of the work on the cognitive effects of unilateral cerebral damage as well as the need for future investigations to take into account the sex of the patients studied.

#### References and Notes

1. A. L. Andersen, *J. Clin. Psychol.* 7, 149 (1951).
2. R. M. Reitan, *J. Comp. Physiol. Psychol.* 48, 474 (1955).

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Cerebral dominance—i.e., greater proficiency of each cerebral hemisphere in acquisition and performance of certain specific functions—has been recognized as a biological characteristic of humans for over 100 years. In most people there is dominance of the left hemisphere for language and of the right side for certain spatial functions. The older literature dealt predominantly with humans, with major stress on the laterality of lesions producing certain cognitive deficits. Since World War II there have been many studies that demonstrate dominance in normals.

By contrast, few studies on laterality deal with the kinds of data that are often found in studies of other biological traits, such as eye color. Examples of such data include the anatomical structures involved, biochemical and immunological properties, genetics, ontogeny, evolution, and comparative features in other species. In older publications one finds a few discussions of associations of handedness, such as asymmetries in fingerprints (1). More recent studies deal with anatomical asymmetries in the human brain, especially in language areas (2–6), asymmetries of function and structure in other species (7–11), the genetics of handedness (10, 12, 13), and the relationship of sinistrality to learning disabilities and to artistic, athletic, and other talents (12, 14). Anatomical maldevelopment has been demonstrated in the left side of the cortex (15) and in the thalamus (16) of a left-handed childhood dyslexic. These studies reflect growing interest in the biological associations of dominance.

Since the autumn of 1980, one of us (N.G.) has been impressed by observations on both patients and normals with an apparently elevated frequency of certain disorders in left-handers and in their families, including immune disorders (e.g., ulcerative colitis, celiac disease, and Hashimoto's thyroiditis) and migraine. Developmental learning disorders (e.g., dyslexia and stuttering) were also present in elevated frequency, as described by others (12, 17). Several family trees of left-handers included, over several generations, many left-handers and individuals with immune disorders, migraine, or learning disorders, either alone or in combination. The decision was made to test these clinical impressions in

studies on left-handers and controls. We also studied the frequency of left-handedness in groups of patients with migraine and immune disorders.

### First Study

#### Methods

For use in our investigation, we developed a questionnaire containing a series of questions concerning personal and family history as well as a modification of the Oldfield Handedness Inventory (18). The score on this inventory is expressed as a laterality quotient (LQ) which ranges in value from +100 (right-handedness in all tasks) to –100 (complete left-handedness). The  $\chi^2$  test was used in all statistical comparisons.

The first study consisted of two separate investigations. In the first, we distributed 500 questionnaires in a shop in London that supplies items for use by left-handers, and we received 253 responses from individuals who had a LQ of –100. Questionnaires were also filled out by unselected individuals from the general population of Glasgow, including civil service applicants, attendants at conferences of teachers and nurses and their spouses, and individuals coming to a shop in Glasgow in which one of our assistants was employed. We selected from this group the questionnaires of 253 individuals, all of whom had a LQ of +100 and who were matched for age and sex to the 253 left-handers.

In the second part of the first study we administered questionnaires to additional individuals gathered in the same manner as the general population group described in the preceding paragraph. Of this group we selected 247 individuals with a LQ of –100 and 647 individuals with a LQ of +100. In the first part of the study we had calculated the frequencies of immune disease entirely on the basis of the subjects' responses to the questionnaire. In the second part we scored a subject as suffering from immune disease only if the diagnosis had been made in a hospital.

#### Results

In part 1 of this study, the frequency of immune disease reported by the left-handed subjects was 2.7

**Table 60.1**  
First study, part 1

	Immune Disorders			Learning Disorders		
	LH	RH	<i>P</i>	LH	RH	<i>P</i>
Subjects*	27 (10.7)	10 (4.0)	<0.005	24 (9.5)	2 (0.8)	<0.005
First-degree relatives†	48	25	<0.01	21	7	<0.01
Second-degree relatives†	45	23	<0.01	11	1	<0.001

LH, left-handers ( $n = 253$ ); RH, right-handers, ( $n = 253$ ).

\*The number and percentage (in parentheses) of subjects suffering from one of the indicated disorders.

†The number of relatives suffering from these disorders.

**Table 60.2**  
First study, part 2

	Immune Disorders			Learning Disorders		
	LH	RH	<i>P</i>	LH	RH	<i>P</i>
Subjects*	13 (5.3)	15 (2.3)	<0.025	27 (10.9)	8 (1.2)	<0.001
First-degree relatives†	58	102	<0.025	19	24	<0.025
Second-degree relatives†	19	18	<0.005	2	4	‡

LH, left-handers ( $n = 247$ ); RH, right-handers ( $n = 647$ ). Footnotes (\*, †) as in Table 1.

‡Numbers too small for calculation of *P*.

times greater than in the control right-handers ( $P < 0.005$ ). The left-handers also reported significantly larger numbers of first- and second-degree relatives with immune disease (table 60.1). Although several types of immune disorders were reported, the frequency of thyroid and bowel disorders was notable. [The following immune disorders were reported: celiac disease, dermatomyositis, diabetes, Hashimoto's thyroiditis, myxedema, regional ileitis (Crohn's disease), rheumatoid arthritis, thyrotoxicosis, ulcerative colitis, and uveitis.]

We also found a much higher frequency of developmental learning disorders (dyslexia and stuttering) reported by the left-handers than by the right-handers ( $P < 0.005$ ). The left-handers also reported significantly larger numbers of relatives with learning disorders (table 60.1).

In part 2 of the first study, the absolute rates of immune disorder in both the left- and right-handers were lower than those in part I because we accepted only those with diagnoses established in a hospital. However, the relative rates were comparable to those found in part 1, with the rate for left-handers 2.3 times as high as that in the right-handers ( $P < 0.025$ ) (table 60.2). Again the left-handers reported significantly higher rates of immune disorders in relatives. The left-handers reported a much higher rate of learning disorders than the right-handers ( $P < 0.001$ ), as well as higher rates in their relatives (table 60.2).

We could not study the frequency of migraine in the two groups because we found that the answers to

questions concerning headache were too vague for proper distinction between migraine and other forms of headache.

## Second Study

### Introduction

In the second study we compared the frequency of left-handedness among patients with migraine or immune disorders seen in the neurological clinics in Glasgow and in a group of general population controls.

We expected the study of the patient groups to give results less clearcut than those of the first study. We did not have available groups of patients with those immune disorders (i.e., involving thyroid or bowel) that our first study had found most often in the left-handers. Second, even the patient groups available to us were much smaller than those of the groups in the two parts of the first study.

### Methods

The patient groups consisted of carefully diagnosed patients with severe migraine or with immune disorders. The control group consisted of 1,142 individuals from the general population of Glasgow, selected by the same methods as those described in the first study. The questionnaire and statistical methods were the same as those used in the first study. We compared the frequencies of individuals with different degrees of left-handedness among the patients and the controls. In the 1,142 controls there were 82 individuals with a LQ < 0

**Table 60.3**

Left-handedness in patient and control groups

LQ	C	M	<i>P</i> *	MG	<i>P</i> *
<0	82 (7.2)	17 (11.6)	<0.1	13 (13.3)	<0.05
<-30	71 (6.2)	17 (11.6)	<0.02	9 (9.2)	<0.3
<-50	59 (5.2)	15 (10.3)	<0.02	7 (7.1)	<0.5

C, controls ( $n = 1,142$ ); M, migraine patients ( $n = 146$ ); MG, myasthenia gravis patients ( $n = 98$ ). Each entry indicates the number and percentage (in parentheses) of controls or patients with a LQ below the level indicated on the left.

\**P*, probability of difference between the patient group and the controls.

(i.e., with all degrees of left-handedness)—i.e., 7.2%, a figure comparable to that found by Oldfield (18) in a Scottish population.

### Results

Table 60.3 summarizes the findings from the comparisons of patient groups and controls. There was a higher percentage of left-handedness among the severe migraine patients. The difference in the frequency of left-handedness was marginally significant ( $P < 0.1$ ) at  $LQ < 0$  and significant at  $LQ < -30$  ( $P < 0.02$ ) and  $< -50$  ( $P < 0.02$ ).

In 98 patients with myasthenia gravis there was an elevated frequency of left-handedness at all three cutoff points, although the difference was significant only at  $LQ < 0$  ( $P < 0.05$ ).

The differences in the frequency of left-handedness found in comparisons of other patient groups (102 patients with rheumatoid arthritis, 168 with mixed-collagen vascular diseases, and 118 with multiple sclerosis) and the general population controls were not statistically significant.

### Discussion

The findings of the first study were in conformity with the hypothesis that immune disorders would be present more often in left-handers and their families than in right-handers. However, the data from this study suggest that the elevated rate is the result of preferential increases in certain forms of immune disorder, especially those involving bowel and gut. The first study is also concordant with the hypothesis of a higher rate of learning disabilities in left-handers and their relatives—a result in conformity with many, although not all, earlier studies (12, 17).

Although we believe that the data on immune disorders and learning disabilities reflect large differences between left-handers and right-handers, the reported frequency differences in relatives may be less reliable. An individual with a learning disability or an immune disorder may be more aware of the presence of these

conditions in relatives than would individuals free of these disorders.

As we have already pointed out, we could not carry out an adequate study of the frequency of migraine in sinistrals because the answers to the questionnaire did not permit reliable distinction between migraine and other forms of headache. However, in the second study, we did find a significantly higher number of left-handers among carefully diagnosed patients with migraine.

We found an elevated frequency of left-handedness among patients with myasthenia gravis that was statistically significant only at  $LQ < 0$ , although it was of the same order of magnitude as the elevation in migraine. We did not find significantly elevated frequencies of left-handedness in the other immune disease groups studied. However, we did not have available large groups of cases with the forms of immune disorder found in greatest numbers in the first study. Furthermore, because the frequency of left-handedness in any one form of immune disease is probably lower than is the frequency of all such diseases among left-handers, much larger patient groups will be required than those which were available to us.

The increased rate of learning disorders among left-handers is not difficult to understand because impairments of the left hemisphere that disturb language functions may also cause a shift of handedness to the right hemisphere (19).

The association of sinistrality, learning disorders, and immune disorders suggests the possibility of a common origin that may help in accounting for certain findings in childhood autism, one of the forms of developmental cognitive disorder. Coleman et al. (20) report that 10% of a group of autistic children suffered from well-diagnosed celiac disease, confirming other studies. A common interpretation is that celiac disease may be one cause of autism. An alternative hypothesis is that celiac disease, linked to HLA-B8 (21), is another manifestation of immune disorder in a group with elevated left-handedness. This interpretation is consistent with an elevated rate of hypothyroidism in parents of autistic children and with an elevated number of relatives with Down's syndrome (22), another condition often accompanied by immune alterations in both the patients and their relatives. (23).

The most surprising finding in this study is the markedly elevated frequency of immune disorders in left-handers and in their relatives as compared to the rates in right-handers and their families. One possible explanation is that immune disorders are the result of stress in patients with learning disorders. This explanation is difficult to accept because immune disorders may be found in elevated frequency among relatives without learning disorders.

Another possible explanation for the association of immune disorders and sinistrality is that of separate genes on the same chromosome, with incomplete penetrance, for left-handedness and immune disease. The association would only indicate joint chromosomal localization and not causal connection. However, there is an alternative explanation based on a hypothesis formulated by one of us (N.G.) on the biological foundations of laterality, of which only a very brief summary can now be given.

The neurons that will occupy the cerebral cortex are formed before 20 weeks of gestation in the central core of the developing brain and then migrate to their future locations (24). The study of fetal brains shows that the hemispheres are asymmetrical during gestation (3, 25). We have found that the gyri and sulci of the cortical convexity usually appear earlier on the right side. Chi et al. (4) have found a larger left planum temporale, part of the language area of Wernicke (2), at 31 weeks of gestation, but the homologous region on the right side develops earlier. Ounsted and Taylor (26) hypothesized that the left hemisphere matures later—especially in males—on the basis of the finding that febrile convulsions were most likely to damage the left temporal lobe in boys in the first year of life.

It is proposed that a major influence that slows growth of the convexity of the left hemisphere in utero is testosterone. This effect will usually be greater in males because the fetal testes secrete testosterone. The effect of testosterone on neuronal development has been documented in such structures as the preoptic nucleus in which it has been shown to cause dimorphism in male and female rats (27–29). Diamond et al. (11) have found the right posterior cortex to be thicker in male rats, and it has been found in our laboratory that the cytoarchitectonic areas in the same region are larger in male rats (unpublished observation). The findings of Goldman and Brown (30) are also consistent with differential cortical maturation in males and females.

Delayed growth in the left hemisphere as a result of testosterone would account for the greater frequency of left-handedness in males. When testosterone effects are more marked and neuronal migration is interfered with to a greater extent, abnormalities in the formation of the left hemisphere will result—especially in males—such as those described by Galaburda and Kemper (15) in the left temporal speech area of a severe childhood dyslexic. This type of effect would account for the much greater incidence of learning disorders in boys.

During fetal life the immune system is also maturing. Testosterone has important suppressive effects on the thymus both in utero and after birth (31, 32). Thus, during periods of increased testosterone effects on left

brain development, maturation of the immune system is also likely to be affected. There are studies that support the hypothesis that the fetal thymus controls development of lymphocytes which are responsible for recognition of self-antigens and thus for prevention of autoimmunity (33, 34). Suppression of thymic growth during fetal life might therefore favor the development of autoimmunity in later life.

One might expect that this hypothesis would imply earlier development and higher frequency of immune disease in males. However, testosterone continues in postnatal life to exert a suppressive effect on the thymus. Thus, certain immune disorders such as myasthenia gravis and lupus erythematosus have a higher incidence in young women and older men (35)—i.e., groups in which testosterone effects are lowest. In the genetically autoimmune New Zealand black mouse (36) the disease develops later in the male. Castration accelerates autoimmunity in the male and testosterone administration slows it in the female.

The hypothesis that testosterone has a major role in the development of immunity is in conformity with several experimental studies. Iványi (37) has shown that several loci on the major histocompatibility complex in the mouse control not only the immune system but also various aspects of male differentiation, including mass of testis and thymus, blood testosterone and testosterone-binding globulin levels, sensitivity to testosterone, and expression of H-Y antigen on thymus. Androgens also control the serum level of the fourth component of complement in mice (38). The minor histocompatibility complex controls  $\beta$ -2-microglobulin which is essential for immune responses; Ohno (39) has proposed that it also controls expression of H-Y antigen which is necessary for development of testes. There is thus an intimate bond between the control of male development and many aspects of the immune system.

One must raise the question as to participation of genetic factors in the familial clustering of left-handedness, immune disorders, and learning disabilities. The hereditary patterns can be explained at least in part by the gene complexes mentioned in the preceding paragraph. We presume that other genes also participate in the joint control of anatomical asymmetry and immunity.

The hypothesis summarized here thus proposes that testosterone slows neuronal development in the left hemisphere, while simultaneously affecting immune development, and thus favoring later immune disorder. Conversely, genes controlling immune responsiveness also regulate testosterone effects.

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Subjects with complete androgen insensitivity (AI) are genetic and gonadal males who are unresponsive to androgens. Consequently, they have a female phenotype despite normal or high male levels of plasma testosterone (Imperato-McGinley et al., 1982), and are feminine in gender and behaviour (Masica et al., 1971).

The relationship of androgens to spatial ability has been a subject of controversy. The specific aim of this study, therefore, was to evaluate subjects with AI to determine the effect of androgen insensitivity on cognitive abilities, with particular attention to tests of visuospatial ability, using the Wechsler Intelligence Scale. The study is unique in that affected AI subjects and control males and females are all members of the largest known kindred with this condition (Imperato-McGinley et al., 1982). Additionally, all subjects were raised in the same town, and the majority still reside there. Those who left town did so in early adulthood. Thus, all study subjects were matched for genetic and sociocultural factors to negate the possible influence of these critical variables on test performance, thereby highlighting the effects of androgen unresponsiveness on cognitive function.

The aetiology of androgen insensitivity in the kindred reported herein is due to an absence of binding to the androgen receptor (receptor negative) (Imperato-McGinley et al., 1982). Pedigree analysis of the kindred demonstrates maternal transmission of the condition, which is compatible with X-linked inheritance (Imperato-McGinley et al., 1982). Linkage analysis studies have localized the androgen receptor gene to the proximal portion of the long arm of the X chromosome between q13–q11 (Imperato-McGinley et al., 1990). Studies with cDNA clones which span the androgen receptor and include the steroid binding domain have not demonstrated any gross gene deletion, suggesting a point mutation as the aetiology of the syndrome in this kindred (Imperato-McGinley et al., 1990).

### Subjects

Thirteen subjects with complete androgen insensitivity, ages 18–76, from the same kindred were studied. The

kindred comes from a small town in the Dominican Republic (Imperato-McGinley et al., 1982). Two AI subjects in their seventies were excluded from the primary analyses because of their age. One left-handed AI subject was also excluded, leaving a sample of 10 AIs for the primary analyses. Five affected subjects in the primary analyses were gonadectomized in adulthood and placed on oestrogen and progesterone therapy. These subjects did not differ in test performance from those who had not been gonadectomized. All affected subjects have a female psychosexual orientation and female gender role (Imperato-McGinley et al., 1982).

All control subjects were right-handed. Control males ( $n = 9$ ) were brothers ( $n = 2$ ) and nephews ( $n = 7$ ) of AI subjects. Control females ( $n = 26$ ) included female siblings ( $n = 9$ ) of AI subjects who had children that were not affected. Other female controls were daughters ( $n = 9$ ) of female siblings of AI subjects, daughters ( $n = 6$ ) of a normal brother of an AI subject, and nieces ( $n = 2$ ) of AI subjects. Subjects were excluded from the primary analysis if they were younger than 15 years of age, older than 60 years of age, had hearing impediments, visual problems, or were left-handed.

The mean age of androgen insensitive subjects was 35.8 years. Control females had a mean age of 26.12 years, and control males had a mean age of 23.44 years. Androgen-insensitive subjects had 7.6 years of education, control females 10.56 years and control males 11.11 years.

Mothers ( $n = 3$ ) of androgen-insensitive subjects who are carriers of the condition were also studied. One of the carrier mothers was left-handed and is the mother of the left-handed AI subject.

### Measurements

The Spanish Version of the Wechsler Intelligence Scale (EIWA) was administered to all subjects by the same psychologist (MP). The test was performed in their homes in the Dominican Republic or in New York city, at the Robert Reid Cabral Children's Hospital in Santo Domingo, Dominican Republic, or at Cornell

University Medical Center. General intellectual scores for assessment of cognitive abilities, reported as Full Scale Intelligence Quotient (FIQ), Verbal Intelligence Quotient (VIQ) and Performance Intelligence Quotient (PIQ), were analysed and compared, as well as the 11 subtests of the EIWA. Comparison was also made utilizing Cohen's cognitive factorial clusters in assessing the (WAIS) subtests (Cohen, 1957), which divided subtests scores into three factors: verbal comprehension (VC); perceptual organization (PO); and freedom from distractibility (FD). VC includes the verbal abilities subtests of information, comprehension, similarities, and vocabulary. PO includes the visuospatial subtests of digit symbol, picture completion, picture arrangement, block design and object assembly, and FD includes the subtests of arithmetic and digit span.

Calculations were based on age-corrected scale scores. The Full Scale IQ score for a hypothetically normally distributed sample that yielded a mean FIQ of  $100 \pm 15$  SD, was used as normative score for comparison. Each subtest score has a mean of  $10 \pm 2.5$ . The Harris test for laterality was administered to all participants.

Assessment of socioeconomic status was carried out utilizing the two-factor index of Hollingshead and Redlich (1958). The subjects were grouped into five social classes utilizing factors of parental occupation and education. All subjects fit into Classes IV and V according to this classification.

## Results

As an initial step, univariate *F*-tests were performed on the FIQ scores of control males, control females and AI subjects, and on their ages and years of education. There were no significant differences between the groups in FIQ or education. Although the AI subjects were somewhat older than the other two groups, age did not correlate significantly with FIQ, VIQ or PIQ. Additionally, the subtest scores of the WAIS are age-scaled and calculation of the IQ scores takes account of age in the analysis.

A two-way repeated measures analysis of variance with scale (VIQ, PIQ scores), as a within-subjects variable and groups as a between-subjects variable, demonstrated a main effect of scale ( $(F_{1,41}) = 42.26$ ,  $P < 0.001$ ), indicating that VIQ scores were higher than PIQ scores, with a marginally significant interaction between group and IQ scale ( $(F_{2,41}) = 2.57$ ,  $P < 0.10$ ). Control females had a larger VIQ-PIQ difference than the control males, but the differences did not reach statistical significance (table 61.1). The VIQ-PIQ difference in the AI group was larger than in the other groups, but also did not reach statistical significance.

**Table 61.1**

Mean IQ scores and age-scaled scores in factors of the Spanish version of the WAIS for three groups of subjects

Measure/Groups	Males ( <i>n</i> = 9)	Females ( <i>n</i> = 25)	AIS ( <i>n</i> = 10)
Full IQ	115.2	108.7	104.1
Verbal IQ	116.8	111.1	109.4
Performance IQ	112.1*	105.2	98.2
Verbal IQ – performance IQ	4.7	5.8	11.2
Cohen's factorial clusters			
Verbal comprehension	12.7	12.4	12.2
Perceptual organization	12.2*†	10.7‡	9.0
Freedom from distractibility	13.1*†	10.8	10.3
Verbal comprehension – perceptual organization	0.5*	1.7	3.2

AIS, Androgen-insensitive subjects.

\* Mean score of males significantly greater than that of androgen-insensitive subjects.

† Mean score of males significantly greater than that of females.

‡ Mean score of females significantly greater than that of androgen-insensitive subjects.

$P < 0.05$  in all cases.

The data were then analysed according to the factorial clusters of Cohen (1957). When the factor scores were used as dependent measures, a multivariate analysis of variance revealed a significant difference between the three groups [ $F(6,76) = 6.59$ ,  $P < 0.01$ ; based on Hotelling's  $T^2$ ]. Subsequent examination of the univariate tests of significance indicated that the groups did not differ significantly on VC [ $F(2,41) = 0.14$ , NS]. However, there were group differences on both PO [ $F(2,41) = 7.87$ ,  $P < 0.01$ ] and FD [ $F(2,41) = 7.73$ ,  $P < 0.01$ ].

Multiple comparisons revealed that control females had significantly lower PO scores than control males. AI subjects had significantly lower PO scores than either control males or females at the 0.05 level (table 61.1, figure 61.2).

When the data on VC and PO for the three groups were considered, a two-way analysis of variance, with factor (VC, PO) as a within-subjects variable and groups as a between-subjects variable, indicated that the difference between VC and PO was highest in AI subjects with females intermediate between control males and AI subjects ( $(F_{2,43}) = 6.00$ ,  $P < 0.01$ ), although only the male-AI difference was significant (table 61.1, figure 61.1).

Male controls exceeded both female controls and AI subjects on FD ( $P < 0.05$ ). There was no significant difference in FD between normal female controls and AI subjects (table 61.1, figure 61.2).

To further analyse the possible influence of education as distinct from that of group membership, a multiple regression analysis was used. Covariance analysis was not appropriate, since the groups were not formed randomly (Pedhazur, 1982). Group membership was

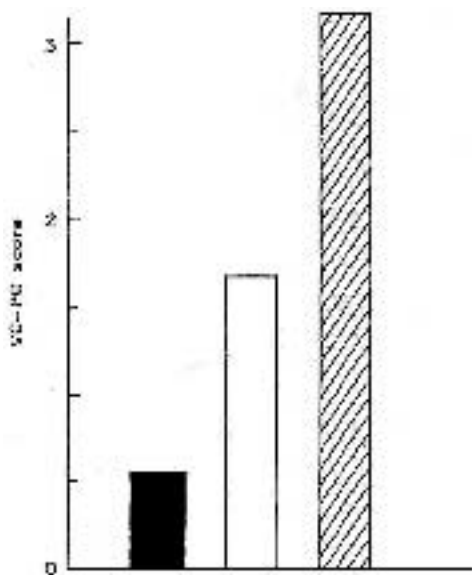


Figure 61.1

Verbal comprehension—perceptual organization scores in ■, males ( $n = 9$ ); □, females ( $n = 25$ ); and ▨, androgen-insensitive subjects (AIS) ( $n = 10$ ). The mean VC-PO score of AIS subjects was significantly greater ( $P < 0.01$ ) than the mean score of normal males.

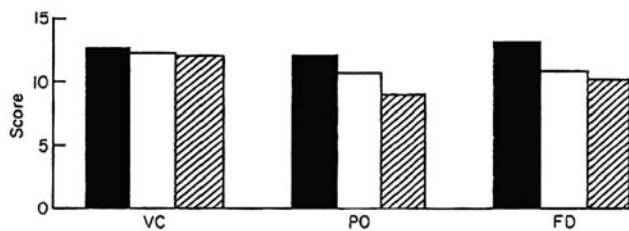


Figure 61.2

Cohen's factorial cluster scores in ■ males ( $n = 9$ ); □, females ( $n = 25$ ); and ▨, androgen-insensitive subjects (AIS) ( $n = 10$ ). The mean score of males was significantly greater than the mean score of female or AIS ( $P < 0.05$ ) on perceptual organization (PO) and freedom from distractibility (FD). The mean female score was significantly greater than the mean AIS score ( $P < 0.05$ ) on perceptual organization.

coded for regression analysis following Pedhazur's effect coding procedure. When the effect of group membership on the VC, PO, and FD clusters, and PIQ was computed, and the effect of education partialled out, the results showed that group membership continued to have a significant effect on PO ( $F(2, 42) = 21.14$ ,  $P < 0.001$ ), FD ( $F(2, 42) = 13.24$ ,  $P < 0.001$ ), and PIQ ( $F(2, 42) = 17.08$ ,  $P < 0.001$ ), indicating that differences in education alone do not explain the pattern of results.

A further multivariate analysis was performed using age-scaled scores from the 11 subtests of the WAIS as dependent variables and revealed a significant effect of group on the canonical variable [ $F(22, 60) = 2.90$ ,  $P < 0.01$ ; based on Hotelling's  $T^2$ ]. Examination of

Table 61.2

Mean age-scaled scores on subtests of the Spanish version of the WAIS for three groups of subjects

Measure/Groups	Males ( $n = 9$ )	Females ( $n = 25$ )	AIS ( $n = 10$ )
<i>Verbal comprehension subtests</i>			
Information	12.4	11.6	11.1
Similarities	12.6	12.8	11.7
Comprehension	12.7	12.2	12.6
Vocabulary	13.2	13.0	13.2
<i>Freedom from distractibility subtests</i>			
Digit span	12.3*	10.8	9.9
Arithmetic	13.9*†	10.9	10.6
<i>Perceptual organization subtests</i>			
Digit symbol	11.8*	11.3‡	8.6
Block design	12.7*†	11.0‡	9.3
Picture arrangement	11.6*	10.3‡	8.2
Picture completion	11.9*†	10.1‡	8.5
Object assembly	13.0*†	10.9	10.2

AIDS, Androgen-insensitive subjects.

\*Mean score of males significantly greater than that of androgen-insensitive subjects.

†Mean score of males significantly greater than that of females.

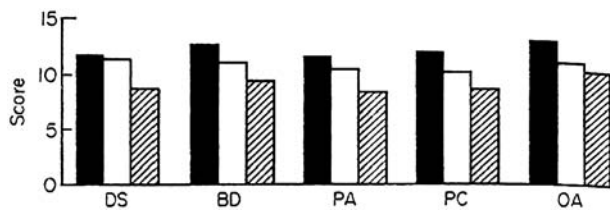
‡Mean score of females significantly greater than that of androgen-insensitive subjects.

$P < 0.05$  in all cases.

the univariate tests of significance indicated that there were significant differences on seven subtests: arithmetic [ $F(2, 41) = 7.43$ ,  $P < 0.01$ ] and digit span [ $F(2, 41) = 3.35$ ,  $P < 0.05$ ] included in the freedom from distractibility factor (FD); and five visuospatial subtests included in the perceptual organization factor (PO); digit symbol [ $F(2, 41) = 7.05$ ,  $P < 0.01$ ]; block design [ $F(2, 41) = 7.84$ ,  $P < 0.01$ ]; picture arrangement [ $F(2, 41) = 4.91$ ,  $P < 0.05$ ]; picture completion [ $F(2, 41) = 7.58$ ,  $P < 0.01$ ]; and object assembly [ $F(2, 41) = 3.35$ ,  $P < 0.05$ ] (table 61.2).

Multiple comparisons using the Neuman-Keuls method at the 0.05 level indicated that males were superior to females on the FD subtest of arithmetic, and the PO subtests of block design, picture completion, and object assembly. Males had significantly higher scores than AI subjects on all seven of the subtests. AIs scored lower than normal females on the subtests of digit symbol, block design, picture arrangement, and picture completion (table 61.2, figure 61.3).

Since eight AI subjects were from two large families within the kindred, their scores were also compared to those of their female siblings. In one family, three AI subjects and three female siblings were tested, and in the second family, five AIs and five female siblings were tested. The two groups were similar in their overall VIQ ( $F(1, 12) = 0.48$ ,  $P = 0.50$ ), although they differed in PIQ ( $F(1, 12) = 4.91$ ,  $P < 0.05$ ). The data from the two families were entered into a three-way analysis of variance with androgen sensitivity and family as between-subjects factors, and the four subtests



**Figure 61.3**

Perceptual organization subtest scores in ■, males ( $n = 9$ ); □, females ( $n = 25$ ); and ▨, androgen-insensitive subjects ( $n = 10$ ). The mean score of males was significantly greater ( $P < 0.05$ ) than the mean score of AIS subjects on all subtests. The mean female score was significantly greater than the mean AIS score ( $P < 0.05$ ) on all subtests except object assembly. DS, Digit symbol; BD, block design; PA, picture arrangement; PC, picture completion; OA, object assembly.

**Table 61.3**

Comparison of androgen-insensitive subjects with female siblings from two families within the large AI kindred, on verbal intelligence quotient, performance intelligence quotient, and four subtests of the Spanish version of the WAIS

	Androgen Insensitives			Female Siblings		
	FAM 1 ( $n = 3$ )	FAM 2 ( $n = 5$ )	AVG ( $n = 8$ )	FAM 1 ( $n = 3$ )	FAM 2 ( $n = 5$ )	AVG ( $n = 8$ )
VIQ	108.3	107.4	107.8	113.0	110.6	111.5
PIQ	93.3	96.6	95.4	105.0	105.6	105.4
DS	8.0	8.6	8.4	8.3	11.2	10.1
PC	7.3	8.4	8.0	9.0	10.8	10.1
BD	9.3	8.4	8.8	10.0	11.2	10.8
PA	6.0	8.8	7.8	9.0	11.8	10.8
Age	42.7	30.8	35.3	50.3	19.4	31.0
Education	8.0	6.4	7.0	8.0	8.8	8.5

VIQ, Verbal IQ; PIQ, performance IQ; DS, digit symbol; PC, picture completion; BD, block design; PA, picture arrangement. FAM, family; AVG, average.

described above as a within-subjects factor. An overall significant difference between AI subjects and their female siblings ( $F(1, 12) = 6.12$ ,  $P < 0.03$ ) was found with no test by androgen insensitivity interaction, indicating that the group difference was consistent across all four subtests (see table 61.3). The analysis also showed that members of family 1 performed somewhat more poorly than members of family 2 ( $F(1, 12) = 3.36$ ,  $P < 0.10$ ), and that there was a family  $\times$  test interaction ( $F(3, 36) = 2.95$ ,  $P < 0.05$ ) with family 1 being particularly poor on picture arrangement and relatively better on block design. The differences between AI subjects and their siblings, however, were consistent across families as well as across tests.

Even though the AIs tended to be the early-born in family 1 and the later-born in family 2, the AI-sibling differences on the PO subtests remained consistent. There were also no significant differences between AIs

**Table 61.4**

A comparison of mean IQ scores, age-scaled scores on Cohen's factorial clusters and subtests of the Spanish version of the WAIS for right-handed androgen-insensitive subjects (AIS), a left-handed androgen-insensitive subject, and two elderly subjects with androgen insensitivity

Measure*/Groups	AIS AIS (Rt Hand) ( $n = 10$ )	AIS AIS (Lft Hand) ( $n = 1$ )	Sub A ( $n = 1$ )	Sub B ( $n = 1$ )
Age	35.2	28	76	73
Education	7.6	14	4	<4
Full scale IQ	104.1	117	105	93
Verbal IQ	109.4	120	118	96
Performance IQ	97.9	112	90	90
Verbal IQ – Performance IQ	11.5	8.0	28.0	6.0
Cohen's factorial clusters				
VC	12.2	14.0	12.5	9.0
PO	9.0	11.8	5.6	5.6
FD	10.3	12.5	13.0	8.5
VC – PO	3.2	2.2	6.9	3.4
Verbal comprehension subtests				
INFO	11.1	15	11	8
SIM	11.7	13	12	8
COMP	12.6	12	14	9
VOC	13.2	16	13	11
Freedom from distractability subtests				
DSp	9.9	13	14	10
ARIT	10.6	12	12	7
Perceptual organization subtests				
DS	8.6	10	2	2
BD	9.3	11	10	9
PA	8.2	12	2	5
PC	8.5	14	6	4
OA	10.2	12	8	8

\*FIQ, Full scale IQ; VIQ, verbal IQ; PIQ, performance IQ; VC, verbal comprehension; PO, perceptual organization; FD, freedom from distractability; INFO, information; SIM, similarities; COMP, comprehension; VOC, vocabulary; DSp, digit span; ARIT, arithmetic; DS, digit symbol; BD, block design; PA, picture arrangement; PC, picture completion; OA, object assembly.

and their female siblings in this subsample on either age or education. Thus, AI subjects show specific deficits on PIQ and four perceptual organization subtests, when compared to the small group of their own female siblings.

A left-handed AI subject and two elderly AI subjects demonstrated the same general basic test pattern as the right-handed subjects (table 61.4). However, the left-handed subject had scores that in general were higher than the mean values of right-handed subjects. Three carrier mothers studied tended to show the same pattern on subtests of visuospatial ability as their AI offspring, i.e., a large VC–PO difference with a poorer performance on the visuospatial subtests included in this cluster (data not shown). However, the sample size was too small for statistical comparison.

## Discussion

Androgens administered early in development to female or castrate male animals have been shown to have a profound effect on adult sexual and nonsexual behaviour. Pre and/or perinatal androgen exposure has been shown to facilitate male response in adulthood and inhibit female response (Paup et al., 1972; Beach, 1975; Goy et al., 1976). Sexual dimorphism in the morphological appearance of certain areas of the brain of various animals has been demonstrated to be due to exposure to male sex hormones at a critical period in gestation (Nottebohm & Arnold, 1976; Gorski et al., 1978).

In humans, sex differences in behaviour and cognition have been well documented. Controversy exists, however, over the role of sex hormone exposure pre and/or postnatally in the mediation of these differences. Consistent differences between the sexes have been found in at least one cognitive function, i.e., males perform consistently better than females in tests of spatial ability, particularly those involving mental rotation (Linn & Petersen, 1985). Male superiority on mathematical ability has been well documented in a Johns Hopkins regional talent search from 1980/1982 where 40,000 gifted seventh graders took the College Board Scholastic Aptitude Test. The results showed a 13-to-1 sex ratio favouring boys in superior mathematical reasoning ability (Benbow & Stanley, 1983).

In the present study, subjects with androgen insensitivity demonstrate a significantly lower performance on the Perceptual Organization factor and subtests of spatial ability than either control males or control females from the same kindred (tables 61.1 and 61.2, figures 61.2 and 61.3).

Additionally, despite the small numbers of study subjects, when female siblings from two families of the kindred were compared with siblings with androgen insensitivity on the same visuospatial subtests, the AI siblings had lower scores (table 61.3).

The androgen-insensitive subjects were raised as females and have a totally female psychosexual orientation. Their cognitive performance, therefore, may reflect their sex roles, as a reflection of social experience and values placed upon them. However, this consideration does not explain their significantly lower overall performance when compared to control females from the same kindred on the perceptual organization factor and subtests of spatial ability. This exaggerated female pattern of performance suggests an effect of androgen unresponsiveness. Furthermore, the fact that there was no difference in performance between AI subjects that were gonadectomized postpubertally and those that were not, suggests that the effect of androgen unre-

sponsiveness on the brain occurred earlier, most likely prenatally or in the early postnatal period.

In the only other study of subjects with androgen insensitivity, Masica et al. (1969) used the Wechsler Adult Intelligence Scale. Male siblings, however, were not studied, and the AI subjects came from seven unrelated families. A modest but consistent and significant tendency toward superiority of VIQ over PIQ was found in 15 subjects with complete androgen insensitivity, similar to their 10 female sibling controls. The androgen-insensitive subjects also demonstrated a lower score on the perceptual organization factor when compared to the verbal comprehension factor. Similar to the present study, the lowest scores were for the visuospatial subtests comprising the perceptual organization factor. There were no comparison data, however, of AI subjects with control males or females on these subtests.

It is worth noting that one of the 13 AI subjects was left-handed. Geschwind and Behan (1982) proposed that left-handedness in males is a consequence of excessive prenatal testosterone levels with earlier maturation of the right hemisphere. The fact that a single genetic and gonadal male with the syndrome of complete androgen insensitivity is left-handed, as well as her carrier mother, challenges a role for androgens in the origin of inherited left-handedness. The overall incidence of left-handedness in our AI population (7.7%) is not significantly different from population estimates in both males and females (Bryden, 1977).

Spatial ability deficits have been consistently noted in other subjects with hormonal deficits in utero. In subjects with Turner's syndrome (45,XO) born with the absence of functional gonadal tissue, verbal IQ was higher than performance IQ. Analysis of the Wechsler Scales according to Cohen's factor scores revealed that the perceptual organization factor (visuospatial tasks) was significantly lower than verbal comprehension, demonstrating an exaggeration of the normal female pattern (Shaffer, 1962; Kolb & Heaton, 1975; Silbert et al., 1977; Gordon & Galatzer, 1980; Money, 1973; Money & Alexander, 1966). These subjects appear also to have additional problems, with tasks not thought to depend primarily on right hemispheric function, which may be related to their chromosomal abnormality (Shaffer, 1962; Silbert et al., 1977).

Studies of men with congenital idiopathic hypogonadotrophic hypogonadism have demonstrated an impairment of spatial ability, as demonstrated by a poor performance on the block design subtest of the WAIS (Hier & Crowley, 1982; Buchsbaum & Henkin, 1980; Teyler et al., 1980). The deficit does not correct with administration of exogenous androgens in adulthood, and does not occur in subjects who develop

hypogonadotrophic hypogonadism following puberty (Hier & Crowley, 1982). Such findings support the present study and suggest that androgens exert a primary organizing effect on the brain, in the prenatal and/or early postnatal period.

### Acknowledgements

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Sex differences in behavior have been documented across species. In humans, certain sex differences in cognitive and emotional processing are increasingly recognized to have biological substrates. Women perform better than men on some verbal tasks, whereas men excel in certain spatial and motor tasks (1). Such differences have been linked to sex hormones (2). In the domain of emotional regulation, men are more likely to express affect instrumentally, such as through physical aggression, whereas women use symbolic mediation, such as through vocal means (3). Women also have a higher incidence of depression (4) and outperform men in emotional discrimination tasks (5). In the human brain, sex differences have been found in the size and morphology of the corpus callosum (6), preoptic anterior hypothalamic areas (7), the bed nucleus of the stria terminalis volume (8), sylvian fissure morphology (9), the percentage of cortical gray matter tissue, and cerebral blood flow (CBF) rate (10).

Theories on brain regulation of human behavior are based primarily on animal experiments, clinical-pathologic correlations, and neurobehavioral studies in healthy persons (11). Studies indicate that emotional processing is primarily regulated by the limbic system and closely related areas (12), and cognitive functions have been linked to neocortical association areas (13). Furthermore, the cerebral hemispheres differ in cognitive and perhaps in emotional processing, with the left specialized for verbal analytic cognition and the right for spatial processing (14). There is also evidence for right hemisphere predominance in emotional processing (15).

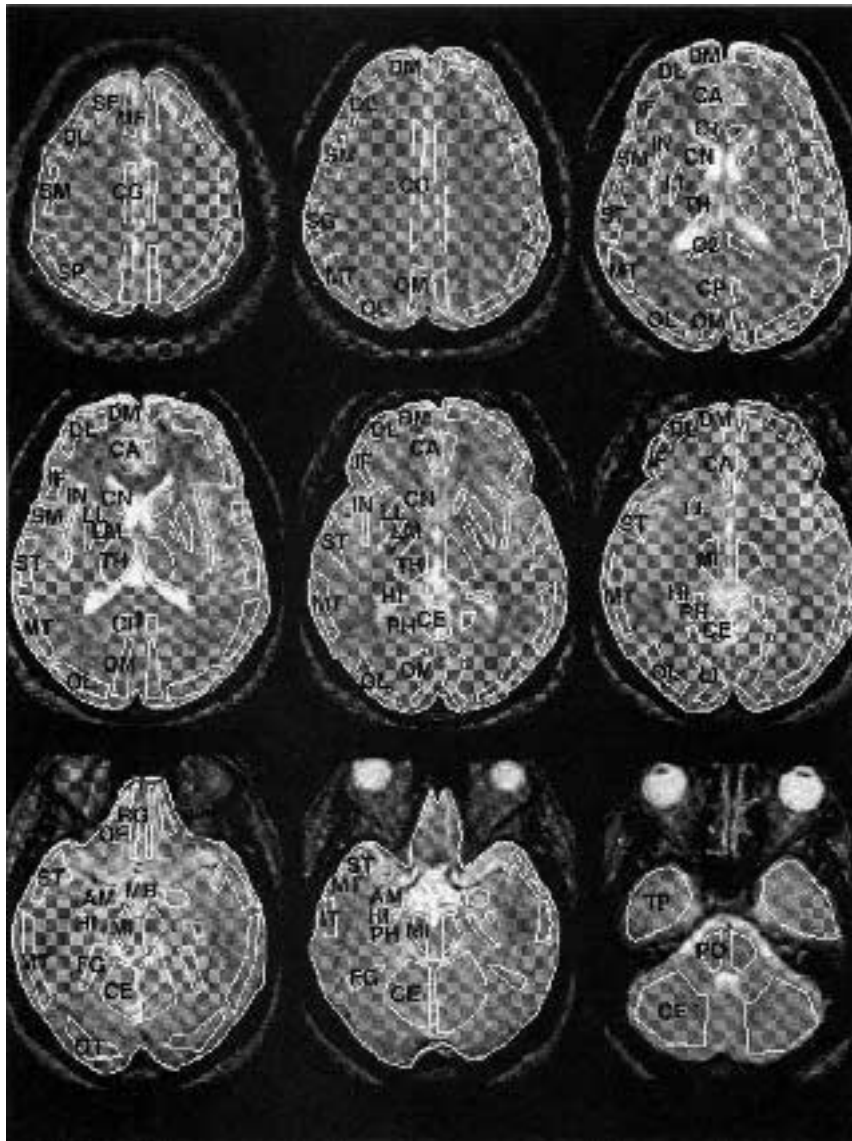
Neuroimaging permits in vivo studies of brain anatomy and physiology pertinent to these issues. Neuroanatomic studies have suggested larger volumes of tissue in left cortical regions implicated in language (16), whereas few systematic asymmetries have been reported in neurophysiologic imaging studies (17). We examined the regional topography of physiologic activity in healthy young adults using positron emission tomography (PET). Our purpose was to evaluate sex differences in the distribution of cerebral metabolism in limbic regions and to investigate systematic asym-

metries that may help us to understand aspects of functional hemispheric specialization.

We studied 61 healthy right-handed volunteers (37 men and 24 women) recruited by advertising. The mean age  $\pm$  SD was  $27.3 \pm 6.5$  years for men and  $27.7 \pm 7.4$  for women, and the mean years of education  $\pm$  SD were  $14.4 \pm 2.0$  and  $14.9 \pm 2.1$ , respectively. Participants underwent comprehensive medical, neurologic, and psychiatric screenings (18). Informed consent was obtained after the nature and possible consequences of the study were explained.

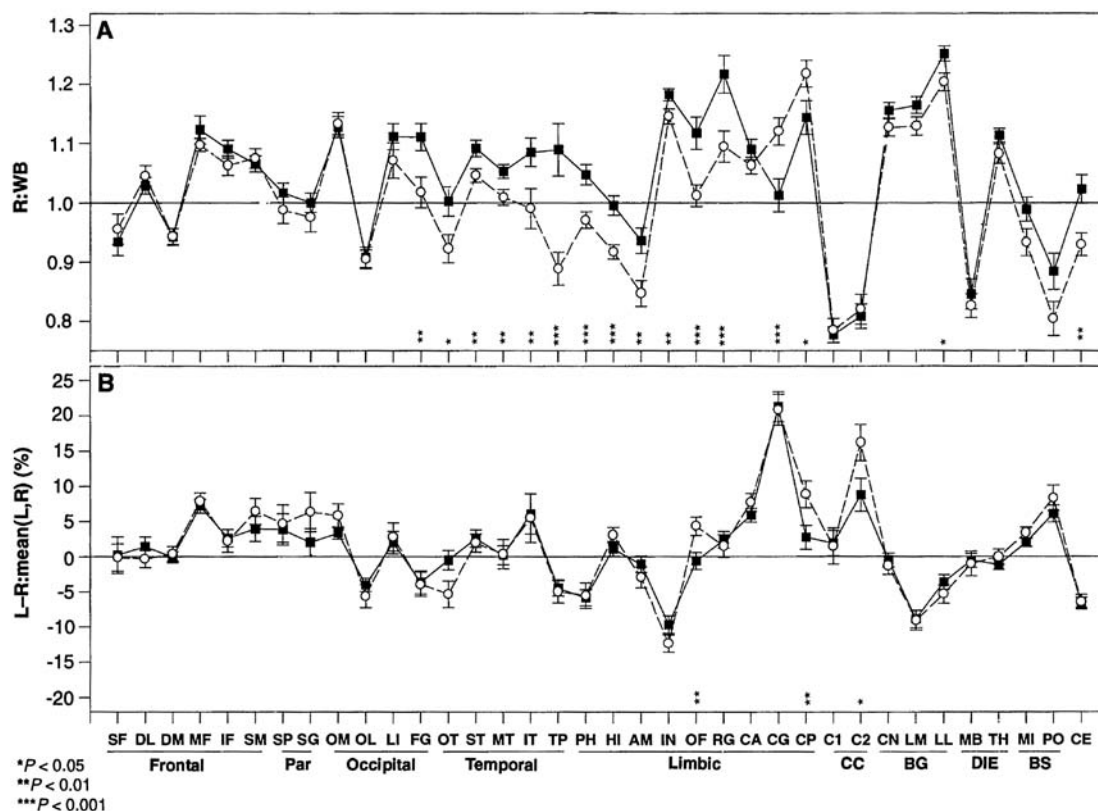
Subjects were scanned after an overnight fast. A radial artery line and a contralateral antebrachium venous line were kept patent with physiological saline. Approximately 185 megabecquerels (5 mCi) of  $^{18}\text{F}$ -labeled 2-fluoro-2-deoxy-D-glucose (FDG) were administered intravenously while subjects lay in a quiet, dimly lit room with eyes open and ears unoccluded. Subjects were instructed to stay quiet and relaxed without either exerting mental effort or falling asleep (19). For determination of the input function, arterial samples were obtained over 90 min. Activity of  $^{18}\text{F}$  in 250- $\mu\text{l}$  aliquots was measured in a dose calibrator after a 3- to 4-hour decay interval. Image acquisition began 40 min after isotope administration with the subjects positioned in a custom-molded, head holder of rigid foam that was aligned by two laser beams situated at right angles. The PENN-PET scanner (20) has a fixed gantry, a 9-cm axial field of view, and an image spatial resolution of 5.5 mm full width at half maximum in all directions. Tissue activity concentration per unit time was calculated with scanner calibration and dead-time correction factors (21). Calibrated blood activity concentration values and metabolism were calculated with lumped and rate constants (22).

We cross-registered PET images with corresponding magnetic resonance images by using established procedures (23). Templates with regions of interest (ROIs) were custom-fitted to magnetic resonance image slices by investigators trained to an interrater reliability criterion of  $>0.85$  (intraclass correlation) for 39 of the 42 regions. Of these, 36 were included in the statistical analysis (figure 62.1). We calculated two indices that



**Figure 62.1**

Placement of ROIs. AM, amygdala; C1, corpus callosum, anterior; C2, corpus callosum, posterior; CA, cingulate gyrus, anterior; CE, cerebellum; CG, cingulate gyrus; CN, caudate nucleus; CP, cingulate gyrus, posterior; DL, dorsal prefrontal, lateral; DM, dorsal prefrontal, medial; FG, fusiform gyrus; HI, hippocampus; IF, inferior frontal; IN, insula; IT, inferior temporal; LI, lingual gyrus; LL, lenticular, lateral (putamen); LM, lenticular, medial (globus pallidus); MB, mammillary body; MF, mid-frontal; MI, midbrain; MT, mid-temporal; OF, orbital frontal; OL, occipital cortex, lateral; OM, occipital cortex, medial; OT, occipital temporal; PH, parahippocampal gyrus; PO, pons; RG, rectal gyrus; SF, superior frontal; SG, supramarginal gyrus; SM, sensorimotor; SP, superior parietal; ST, superior temporal; TH, thalamus; and TP, temporal pole.



**Figure 62.2**

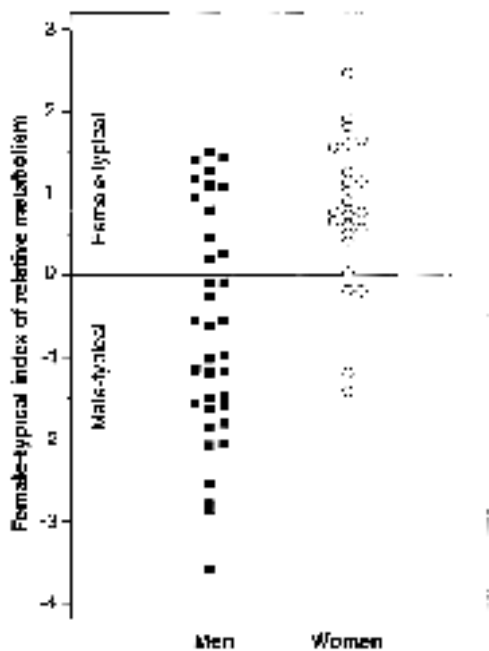
Topography of metabolic activity in men (*filled squares*) and women (*open circles*) for the ROIs delineated in figure 62.1. Cortical regions are grouped by lobe in a rostral to caudal order [frontal, parietal (Par), occipital, temporal, limbic] followed by corpus callosum (CC) and subcortical regions [basal ganglia (BG), diencephalon (DIE), and brainstem (BS)]. Abbreviations are as in figure 62.1. This order heuristically reflects ontogenic and evolutionary development. (A) Means  $\pm$  SEM of R:WB ratios. (B) Means  $\pm$  SEM of laterality differences in percentages. L, left; R, right.

are based on count rates: (i) region: whole brain (R:WB) ratios averaging the two hemispheres and (ii) a laterality index [percent (left minus right):mean (left, right)].

Average metabolism calculated from WB counts did not differ between men (mean  $\pm$  SD,  $4.66 \pm 0.97$  ml per 100 g of tissue mass per minute) and women ( $4.62 \pm 1.09$ ),  $t(58) < 1$ . This is consistent with cerebral glucose metabolism studies (24) but contrasts with studies of CBF, where women have higher rates. Analysis of the regional distribution of metabolic activity (figure 62.2A) revealed that, in both men and women, metabolism was lowest in the corpus callosum and highest in the basal ganglia (25). A sex  $\times$  region analysis of variance (testing the hypothesis of gender differences in regional metabolism) yielded a sex  $\times$  region interaction of  $F(35, 2065) = 3.55$ ,  $P < 0.001$ . The groups had identical relative metabolism in all nonlimbic frontal, parietal, and occipital regions, but sex differences were prevalent in temporal-limbic regions, basal ganglia, brainstem, and cerebellum. Whereas men had higher relative metabolism in lateral

and ventro-medial aspects of temporal lobe regions, they had lower relative metabolism in the middle and posterior cingulate gyrus. The number of regions showing significant sex differences (17) is larger than would be expected by chance ( $2/36$  at the 0.05 level),  $z = 10.6$ ,  $P < 0.0001$ . The raw absolute metabolic rates were only higher in men than in women for the occipital temporal region, temporal pole, hippocampus, amygdala, and orbital frontal cortex ( $P < 0.05$ ).

To examine individual differences in this pattern, we calculated a "female-typical" score by standardizing ( $z$  transformation; mean = 0, SD = 1) the regional metabolic rates and subtracting metabolism in the temporal-limbic regions from cingulate metabolism (figure 62.3). Only 17 of the 61 subjects (13 men and 4 women) had scores opposite to their respective sex-typical indices,  $\chi^2(1) = 12.7$ ,  $P < 0.001$ . This could not be explained by sex differences in anxiety because the two groups did not differ in reported anxiety (26), either for trait (men,  $32.4 \pm 6.9$ ; women,  $32.2 \pm 7.8$ ),  $t(59) < 1$ , or state ( $34.6 \pm 6.8$  and  $36.6 \pm 6.9$ , respectively),  $t = 1.09$  (27).



**Figure 62.3**

Scatterplot showing individual differences in the “female-typical” index of relative metabolism with temporal-limbic data subtracted from cingulate averages.

Examination of the laterality of metabolism indicated that asymmetries were ubiquitous in both men and women (figure 62.2B). Of 36 regions, 20 deviated from symmetry at the 0.01 significance level, with five additional differences significant at  $P = 0.05$ . Some of the asymmetries seem related to functional brain organization. Consistent with the dextrality of our subjects, metabolism was relatively higher in the left hemisphere for premotor, motor, and sensorimotor cortex and both brainstem regions (midbrain and pons), which control contralateral motor functions, and higher in the right for cerebellum, which influences motor functions ipsilaterally. The higher left hemispheric values observed in medial and inferior frontal, parietal, superior, and inferior temporal cortices and cingulate gyrus may reflect the role of these regions in verbal-analytic functions. By contrast, higher right hemispheric metabolism was evident in most ventro-medial temporal lobe components of the limbic system and their projections in lenticular regions of the basal ganglia, consistent with theories of right hemispheric predominance for emotional processing. Men and women were nearly identical in the topographic distribution of lateral asymmetries, with only three regions showing sex differences at the 0.01 level. These regions were the orbital-frontal, posterior cingulate, and posterior corpus callosum, where women had relatively higher left hemispheric metabolism than men.

The findings indicate sex differences in the regional topography of resting cerebral metabolic activity and systematic asymmetries in regional metabolism that are largely shared by men and women. The higher relative metabolism in men in the temporal-limbic system and the reversal of this difference for the middle and posterior cingulate gyrus, where women had higher metabolism, may reflect a different baseline activity. This could relate to emotional processing modes because the cingulate is cytoarchitecturally one of the more complex components of the limbic system.

The observed regional variation in hemispheric asymmetries suggests greater left hemispheric activity in neocortical regions and cingulate cortex and higher right hemispheric metabolism in limbic regions of the ventro-medial temporal lobe, closely associated occipital-temporal cortices, and ventral striatum. Sex differences in these asymmetries were limited and could be chance effects because they appeared in few regions. The predominance of lateral asymmetry in regional cerebral metabolism contrasts with the paucity of lateral asymmetries found in anatomic studies, as well as in physiologic investigations with other methods. The relatively higher left hemispheric activity in premotor, primary motor, and somatosensory regions is consistent with reports of neuroanatomic studies (28). However, we found higher right hemispheric metabolism in the medial and lateral lenticular regions, where an anatomic study had reported higher left hemispheric volumes (29), and symmetric metabolism in the caudate nucleus, where higher right hemispheric volume had been reported. Possibly, correlations between volume and metabolism differ among regions. The larger number of asymmetric regions relative to earlier neurophysiologic studies may reflect improved resolution and analysis methods and larger samples. The  $^{133}\text{Xe}$  clearance studies were sensitive to activity only in superficial layers of cortex, whereas tomographic studies with smaller sample size (particularly of women) did report some asymmetries (24).

Although we have focused our attention on hemispheric asymmetry and sex differences, these differences in specific regions occur against a background of similarities in the profile of metabolic activity, both between the two hemispheres and between men and women. This similarity attests to the overall reliability of the metabolic parameters and also reminds us that the two cerebral hemispheres and the brains of men and women are fundamentally more similar than different.

Our study was limited to examining the resting state and did not incorporate activation procedures. Thus, our conclusions concern topography of the human brain while it is “idling” in a semistructured environ-

ment, which may itself influence regional brain activity. Further regional and sex differences may become evident when activity is measured during the performance of behavioral tasks or pharmacologic challenges. Nonetheless, the results suggest neural substrates for domains of human behavior related to both cognitive and emotional processing. They support a neurobiologic explanation of some sex differences in these behavioral dimensions and thus may help to explain sex-related differences in behavior. Individual differences within a sex and the overlap between the sexes may reflect "noise" in the measurement but perhaps, as can be tested empirically, can also be related to individual differences in sex-typical behavior.

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It is now generally agreed that males and females probably differ in brain organization for intellectual or problem-solving behaviours. There is less agreement on the nature of such sex differences, but the trend in recent years has been to emphasize that males show greater functional asymmetry between left and right hemispheres than do females (Harshman & Remington, 1976; Kimura, 1969; Lake & Bryden, 1976; Lansdell, 1962). Gross speech deficits (aphasia), as well as subtler deficits in language measured by verbal intelligence tests, have been reported to be more tightly associated with left-hemisphere damage in males than in females (McGlone, 1977, 1980).

Neurological damage, when it occurs spontaneously in humans through strokes or tumors, tends to involve much of one hemisphere, and may often leave the other hemisphere relatively intact. This fact has led to widespread comparisons of the effects of undifferentiated left-hemisphere damage with undifferentiated right-hemisphere damage; while this is a legitimate procedure, it necessarily presents only a crude picture of brain organization. In the course of further subdividing our patient groups with unilateral brain damage into those with more restricted lesions of various areas, it became apparent that there were some interesting sex differences in the dependence of speech function on anterior or posterior regions of the left hemisphere (Kimura, 1980). This paper reports in detail the retrospective data from our total population to date.

## **Method and Results**

### **Left-Hemisphere Lesions**

The pool of subjects was a total of 216 right-handed patients with damage restricted to the left hemisphere, who were seen by the neuropsychology service at University Hospital since 1973. Most patients were seen within two weeks of admission for acute symptoms related to their neurological problems. The only criterion for selection was that damage be unilateral, as determined by all available medical evidence (see below). In the last 5 or 6 years at least, most patients with unilateral damage who came through the wards would

have been included in this sample. Patients were identified as aphasic by a criterion of either grossly apparent dysphasia (some of the early cases), or by a cutoff score on a standard test of aphasia which samples both expressive and receptive function (Mateer & Kimura, 1977), or by a cutoff score on an abbreviated version of the Token test (Kimura & McGlone, 1983; de Renzi & Vignolo, 1962). Of the 143 males, 73 or 51% were aphasic by these criteria and of the 73 females, only 22 or 31% were aphasic. These findings essentially confirm McGlone's (1977, 1980) report, and indeed the patient and test data overlap considerably with hers; that is, all of McGlone's cases are included in the present study, and form about 20% of the sample.

If one looks at the clinical characteristics of males and females with left-hemisphere damage, there do not appear to be any significant differences in neurological symptomatology apart from the incidence of aphasia (table 63.1). There is, however, a significantly higher proportion of vascular accidents in the male than the female sample, consistent with some studies (Hutchinson & Acheson, 1975). There is no significant difference in proportion of tumours. A small number of cases in each group have seizures due to atrophic lesions.

For the aphasic subgroups, general characteristics of the type and severity of the disorder are given in table 63.2. Fluency, for which scores are available only since 1976, is measured as the mean number of utterances per phrase. The Token test is a locally developed abbreviated version of a nonredundant speech comprehension test introduced by de Renzi and Vignolo (1962). There are no significant differences between males and females on any of these measures, but there is a trend for fluency to be lower in female aphasics. This finding contrasts with Brust, Shafer, Richter, and Bruun's (1976) claim of greater nonfluency in male aphasics, but the trend in Brust et al.'s data did not approach statistical significance.

Eighty-one of the original 216 subjects with unilateral left-hemisphere damage could be further subdivided into those with lesions confined either to anterior left hemisphere, in front of the Rolandic

**Table 63.1**Clinical information on patients with left-hemisphere damage ( $N = 216$ )

	<i>N</i>	Age	# Hemiparesis	# Field Defects	# Tumours	# Vascular	# Aphasia
Male	143	50.4	43 (29%)	38 (27%)	37 (26%)	97 (68%)	73 (51%)
Female	73	45.7	21 (29%)	18 (25%)	24 (33%)	38 (52%)	22 (31%)
M/F, $\chi^2$ ( $df = 1$ ):			.0085	.0924	1.1694	5.1329*	8.5781**

\* $p < .05$ ; \*\* $p < .01$ .**Table 63.2**Characteristics of aphasics with unilateral left-hemisphere damage ( $N = 95$ )

	Males (73)	Females (22)	M/F	<i>df</i>
# Vascular	59 (81%)	14 (64%)	$\chi^2 = 2.5104$	1
# Hemiparesis	38 (52%)	13 (59%)	$\chi^2 = .0035$	1
# Field Defects	30 (41%)	9 (41%)	$\chi^2 = .0924$	1
# Fluent	34/58	5/15	$\chi^2 = 3.0627^\dagger$	1
Receptive Score <sup>a</sup>	30.2	31.4	$t = .199$	88
Expressive Score <sup>a</sup>	30.8	27.9	$t = .481$	88
Token Score <sup>a</sup>	48.4	52.6	$t = .452$	44

<sup>a</sup>These measures are described in detail in Kimura and McGlone (1983).<sup>†</sup> $p < .10$ .

fissure, or to posterior left hemisphere, behind the Rolandic fissure and including the temporal lobe (see figure 63.1 for source of subgroups). Such classification had been made retrospectively and independent of psychological test data. Anterior-posterior classification utilized information from the neurological examination (presence of field defects, hemiparesis), angiography, CT scan, EEG, brain scan, etc. Where extent or locus of lesion was unclear from reports in the medical charts, the films were viewed directly, and in cases in which there still was doubt, the neuroradiologist was consulted to determine locus from the angiogram or CT scan. Of these 81 patients, 49 were male and 32 female. Data on neurological symptoms and etiology are given in table 63.3.

Of the 49 males, 20 or 41% were aphasic, and of the 32 females, 10 or 31% were aphasic. It is apparent in this group with restricted lesions, that vascular accidents are less common than in the larger pool from which they were selected (see table 63.1). The incidence of tumours and vascular accidents is approximately equal here, and very similar in males and females. There are no significant differences between males and females on any of the measures reported in table 63.3, including aphasia, although the trend for aphasia to be more frequent in males is maintained.

However, when one examines the effects of locus of damage within the hemisphere on incidence of aphasia, a striking sex difference emerges. Of the 20 aphasic

males, 14 or 70% owe their aphasia to a posterior lesion, whereas of the 10 aphasic females, 8 of the 10 or 80% owe their aphasia to an anterior lesion (table 63.4). The pattern is significantly different from that predicted by chance [ $N = 81$ ,  $\chi^2$  for the 3-way interaction of locus (anterior, posterior), sex (male, female) and aphasia (aphasic, nonaphasic), using a  $2 \times 2 \times 2$  contingency table = 5.6006;  $df = 1$ ,  $p < .02$ ].

The overall incidence of restricted posterior damage is higher in our sample than is restricted anterior damage, due possibly to the larger area encompassed by the "posterior" classification, and to the fact that restricted vascular lesions in our sample tended to affect posterior regions more often than anterior. The finding of a higher incidence of aphasia after anterior damage in females is therefore particularly noteworthy. In fact, the interaction between locus of lesion and aphasia is due almost entirely to the females. This is demonstrated by the fact that tests for independence of locus and incidence of aphasia depart significantly from chance for females when analyzed alone ( $\chi^2 = 9.3491$ ,  $df = 1$ ,  $p < .01$ ), but not for males when analyzed alone ( $\chi^2 = .006$ , *ns*). Thus there is a proportionally higher incidence of aphasia from anterior than from posterior lesions in females, but this is not true for males.

**Etiology** This pattern is not explicable on the basis of a sex difference in etiology of damage. The proportion of tumours to vascular accidents is roughly the same in males and females with restricted lesions (table 63.3). Moreover, if one looks at tumours alone, or vascular accidents alone, a sex difference emerges in each case. The pattern of sex differences is quite comparable in the tumour and vascular groups, but the overall incidence of aphasia in the tumour cases (35%) is slightly lower than in the vascular cases (43%).

**Locus of Damage within the Posterior Region** The question arises whether the extremely low incidence of aphasia after posterior lesions in females is due to the fact that the critical speech zones were unaffected. If one selects those patients who have brain damage unequivocally involving posterior superior temporal and/or anterior parietal lobes, in the posterior peri-Sylvian region, the data indicate that this region can be damaged with lesser effects on speech in females than in

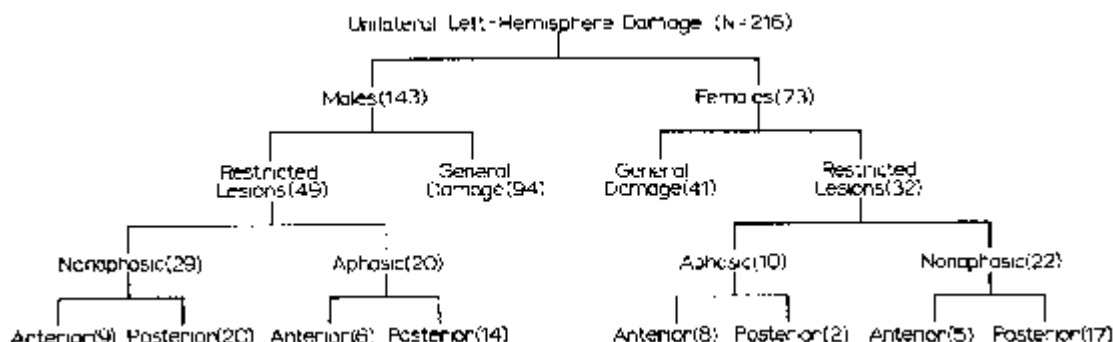


Figure 63.1

Source of aphasic patients with restricted left-hemisphere lesions

Table 63.3

Characteristics of patients with restricted left-hemisphere damage ( $N = 81$ )

	<i>N</i>	Age	# Vascular	# Tumour	# Hemiparesis	# Field Def.	# Aphasia
Males	49	47.2	25 (51%)	22 (45%)	3 (6%)	15 (31%)	20 (41%)
Females	32	47.0	15 (47%)	15 (47%)	4 (13%)	7 (22%)	10 (31%)
M/F, $\chi^2$ ( $df = 1$ ):			.1331	.0305	.9973	.7470	.7847

Table 63.4

Incidence of aphasia in restricted left-hemisphere lesions ( $N = 81$ )

	Total Sample	# Aphasic	Age	# Non-Aphasic	Age
<i>Anterior Lesions</i>					
Males	15	6 (40%)	53.5	9	46.2
Females	13	8 (62%)	57.9	5	57.8
<i>Posterior Lesions</i>					
Males	34	14 (41%)	56.4	20	39.2
Females	19	2 (11%)	49.0	17	38.5
$\chi^2$ (Sex $\times$ Locus $\times$ Aphasia) = 5.6006, $df = 1$ , $p < .02$					

males. Thus, of 9 females with such damage, only 2 were aphasic, while of 18 males, 13 were aphasic ( $\chi^2 = 6.075$ ,  $df = 1$ ,  $p < .02$ ). In particular, parietal-lobe damage by itself has not resulted in aphasia in any females to date ( $N = 6$ ), while of the 10 cases of parietal damage in males, 7 are aphasic. One must conclude that the evidence in the literature for the critical importance of this region for speech has rested on data from males, who outnumber females almost 2 to 1 in our total sample.

The possibility remains that in females speech depends critically on some other areas in the left posterior region, or that it is more diffusely organized in this region. This hypothesis receives no support from the fact that in the only 2 cases of aphasia from posterior damage, the area primarily affected happened to be fairly restricted to superior and posterior temporal areas and corresponded roughly to Wernicke's area. A more probable inference is that some of the functions

vested in the posterior region in males are mediated by anterior systems in females, more evidence for which will be presented in the section on apraxia.

**Locus of Damage within the Anterior Region** Locus of damage within the anterior region in the aphasic patients tended to be fairly extensive, and the extent of damage as evidenced from CT scans and angiograms appeared quite comparable in males and females. It is of passing interest to note that not all lesions in aphasics involved Broca's area directly, and in at least two cases apparently did not impinge on this area even indirectly. Because the number of patients with damage restricted to the anterior region is smaller than the number with posterior lesions, more specific statements about the relationships between locus of lesion within the anterior region, and deficits on speech and praxis tasks, will have to wait until more data are collected.

**Age** The mean age for each subgroup in the present study is given in table 63.4. It can be seen that the aphasic groups as a whole are older than the non-aphasics. However, the difference between males and females in susceptibility to aphasia is not readily explicable on the basis of age differences, since males and females with posterior damage have equivalent ages. Nevertheless, to ensure that age was not a determining factor, only data for patients over age 40 was analyzed separately. A  $\chi^2$  test for the 3-way interaction of sex, locus, and incidence of aphasia in patients over 40 yields a value of 4.1275 ( $df = 1$ ,  $p < .05$ ). It thus appears that the differential sensitivity of males and

**Table 63.5**Mean speech scores in aphasic patients with restricted left-hemisphere lesions ( $N = 30$ )

	<i>N</i>	Receptive/43	Expressive/47	Token/84	Fluency
Male Anterior	6	34.0 (79%)	36.2 (77%)	54.8 (65%)	7.4
Male Posterior	14	31.9 (74%)	35.1 (75%)	59.3 (71%)	12.1
Female Anterior	8	30.9 (72%)	33.9 (72%)	58.5 (70%)	6.3
Female Posterior	2	39.0 (91%)	37.5 (80%)	65.5 (78%)	9.9

females to locus of damage in producing aphasia is not due to age differences.

**Severity of Aphasia** If the left anterior part of the brain is indeed predominant over the posterior for speech functions in females, one might expect some qualitative effects of damage here, which are absent in males. In support of this idea, a perusal of the aphasia scores (table 63.5) suggests that in females a left anterior lesion uniformly produces a more severe speech disturbance than does a posterior lesion. No such consistent pattern emerges for males except for fluency. (Fluency has generally been found more depressed by anterior lesions than by posterior lesions, e.g., Benson, 1967.) The sample size in females with posterior damage is so small, however, that no statistical comparisons of scores were made between anterior and posterior aphasics.

**Apraxia** There is a tendency for a left anterior lesion in a female to produce a composite of the deficits typically seen after anterior or posterior damage in males. This is particularly evident in aphasic females, but it holds even for nonaphasics (table 63.6). Thus the group of females with left anterior damage shows not only speech disorders, and the associated difficulty in making nonverbal oral movements typical of anterior damage, but there is also a difficulty in copying manual movements, which in males is most readily seen after parietal-lobe damage (Kimura, 1982). Moreover, there is a very marked difficulty in females with anterior lesions on constructional tasks such as the Block Design subtest of the WAIS (Wechsler, 1955).

Of the two female aphasics with *posterior* damage, one had absolutely no difficulty in the production of nonverbal oral movements (a score of 8 out of 9), nor on manual apraxia (22 out of 24) or constructional tests. The degree of this dissociation was beyond that seen in any other aphasic patient. The other patient surpassed all but one of the male posterior aphasics in copying manual movements, but she did show difficulty with multiple oral movements. Of the 6 females with restricted parietal-lobe damage (none of whom were aphasic), only one had any difficulty with constructional or manual tasks, whereas of the 10 males

**Table 63.6**Praxis scores in patients with restricted left-hemisphere lesions ( $N = 81$ )

	<i>N</i>	Multiple Oral (Max = 9)	Multiple Manual (L hand, Max = 24)	Block Design (Max = 48)
<i>Males</i>				
Anterior	15	4.6	15.3	21.5
Posterior	34	5.1	13.1	23.5
<i>Females</i>				
Anterior	13	4.6	9.9	10.8
Posterior	19	6.5	18.1	28.9 <sup>a</sup>
Aphasics Only ( $N = 30$ )				
<i>Males</i>				
Anterior	6	2.2	12.8	18.5
Posterior	14	3.7	9.2	20.2
<i>Females</i>				
Anterior	8	2.6	8.3	7.7
Posterior	2	5.0	18.5 <sup>a</sup>	32.0 <sup>a</sup>

<sup>a</sup>Essentially normal scores.

with such damage, 7 had unequivocal and usually severe difficulty with these tasks. Not only speech but also oral and manual praxis appear to be critically dependent on the anterior left hemisphere in females, but in males may be affected by damage to either anterior or posterior regions on the left. In fact, the data from table 63.6 suggest that manual praxis and the constructional ability associated with the latter, may be even more vested in the anterior region in a female than are the more narrowly speech-related skills (table 63.5).

### Right-Hemisphere Lesions

**Incidence of Right-Hemisphere Speech** Ninety males and 79 females with unilateral lesions of the right hemisphere, and with right-hand preference, had been seen in the research series. The criterion for inclusion again was simply that damage be unilateral. Two of the 90 males (2%) and 1 of the 79 females (1%) were aphasic as a consequence of right-hemisphere damage, obviously a very low incidence in both groups. Viewing the data another way, aphasia in males was a consequence of left-hemisphere damage 73 times, and of

**Table 63.7**  
IQ scores in patients with unilateral right-hemisphere lesions

	<i>N</i>	Verbal IQ	<i>SD</i>	Perfor- mance IQ	<i>SD</i>	<i>V - P</i>
Males	87	104.6	15.24	93.2	17.26	11.4
Females	72	98.7	13.76	94.5	14.99	4.2
M/F:		$t = 2.61, p < .01$		$t = .508$		

right-hemisphere damage twice (3% of total). In females, aphasia resulted from left-hemisphere damage 22 times and from right-hemisphere damage once (4% of total). These incidences clearly do not differ.

Even in left-handed and ambidextrous patients, there is no trend for speech to be more often affected by right-hemisphere damage in females. In addition to our total sample of 385 right-handed patients, we tested 50 patients who were left-handed or ambidextrous. This number includes all left-handed or ambidextrous patients, regardless of unilaterality of damage. Four of the 23 females were aphasic, 3 from left-hemisphere damage and 1 from bilateral damage; 3 of the 27 males were aphasic, 2 from left-hemisphere damage and 1 from bilateral damage.

It appears that, at least with adult lesions, when patients are selected on a criterion other than aphasia, the incidence of unilateral right-hemisphere speech representation is very low. Moreover, it is clearly not different between males and females.

#### Verbal Intelligence after Right-Hemisphere Lesions

Seventy-two of the 79 females and 87 of the 90 males with right-hemisphere damage had been administered both the Verbal and Performance Scales of the Wechsler Adult Intelligence Scale (Wechsler, 1955). The data are shown in table 63.7. The depression of the Performance IQ relative to the Verbal IQ in males is consistent with McGlone's (1978) report. However, the critical fact in favour of greater right-hemisphere participation in verbal functions in females than in males would be a lower Verbal IQ in females than in males. There certainly appears to be such a difference in McGlone's reports (1977, 1980), but her claim that the Verbal IQ is depressed after right-hemisphere lesions in females was instead based on a comparison with normal subjects, who had an overall higher IQ than any of the brain-damaged groups. In the present study, no such comparison with normals was made, since, for both male and female patient groups, the Verbal IQ means are near the population average of 100. When males and females are compared on Verbal IQ alone, the difference between the means, although not large, is statistically significant ( $t = 2.61, df = 157, p < .01$ ).

The Performance IQ's do not differ significantly ( $t = .508$ ).

#### Intrahemispheric Organization of Constructional Ability in the Right Hemisphere

Because of the very strong evidence of anterior-posterior differences across sexes in speech and praxic function, it seemed worthwhile to search for a parallel organization in the right-hemisphere. Accordingly, patients with damage restricted to anterior or posterior regions were examined for performance on nonverbal, particularly constructional, tasks. Of the 169 patients with unilateral right-hemisphere damage, 53 had restricted lesions. The sample size is considerably smaller than for the left-hemisphere lesion groups, so the scores presented in table 63.8 must be considered preliminary. In addition to overall Verbal and Performance IQ measures, data from two clearly constructional tasks from the WAIS—Block Design and Object Assembly—are also given. Within-sex comparisons show no significant differences on any measure except for the Block Design score between the two female groups ( $t = 2.11, df = 23, p < .05$ ), but there is also a trend towards a difference on the Object Assembly subtest ( $t = 2.06, df = 23, P < .06$ ). Obviously, with the very small sample in the right anterior female group, any inferences concerning effect of locus on performance scores must be tentative, yet the parallel to the effect of restricted left-hemisphere lesions on Block Design is rather striking (table 63.6). Since the right-hemisphere group is an entirely different sample, the trends in their data add strength to the suggestion of sex differences in the organization of function along anterior-posterior dimensions.

In contrast to their impaired performance on constructional tasks, not a single one of the 6 women with right anterior damage had any difficulty in copying manual movements (range 18–23, table 63.8). In the left anterior group of females, all but 2 of the 13 had significant difficulty in performing this task (whether aphasic or not), and even in these two, the scores were not normal (range 0–16, table 63.6). In other words, there is no overlap in scores on manual praxis between left and right female anterior groups.

#### Discussion

The fact that aphasia and apraxia in females occur more often from anterior than from posterior damage to the left hemisphere, suggests that the underlying functions are organized differently in the brains of normal females and males. It appears that in females, speech and the associated oral and manual praxic functions (Kimura, 1982) are more dependent on the

**Table 63.8**Effect of right-hemisphere lesions on performance IQ and manual praxis measures ( $N = 53$ )

	<i>N</i>	Verbal IQ	Performance IQ	Block Design (Max = 48)	Object Assembly (Max = 44)	Multiple Manual (Max = 24)
<i>Males</i>						
Anterior	11	106.6	100.9	27.3	27.5	18.3
Posterior	16	104.4	95.1	26.4	21.6	17.9
Ant/Post <i>t</i> =		.442	.881	.158	1.48	.300
<i>Females</i>						
Anterior	6	96.0	91.2	20.3	19.0	19.8 <sup>a</sup>
Posterior	20	99.7	98.8	29.3 <sup>a</sup>	26.4	19.1 <sup>a</sup>
Ant/Post <i>t</i> =		.688	1.36	2.11*	2.06 <sup>†</sup>	.454

<sup>a</sup> Essentially normal scores.\*  $p < .05$ .<sup>†</sup>  $p < .06$ .

anterior part of the left hemisphere than on the posterior. This is not true for males, where the two regions contribute more equally than in females, or if anything, show the reverse pattern. Why this sex difference has previously gone undetected is rather a puzzle, but it is probably explicable on the basis that it has not previously been studied. Comparing the incidence of aphasia after various lesions requires that one record not only the aphasic patients, but also the nonaphasic. If one studies only patients with aphasia (or indeed any group chosen on the basis of a particular symptomatology), one cannot reliably make statements about the probability that a particular lesion will produce the symptom in question, since there is no way of knowing how many patients with this lesion do not show the symptom. This point was effectively made by von Monakow with respect to apraxia many years ago (1914), but it has often been lost sight of in the case report method. Even comparing the relative efficacy of two different lesions in producing a symptom is not possible unless one knows how often each of these two types of lesions occurs in the population, and again this implies selection on some basis other than symptomatology.

In the present study, aphasia was more frequent in males than in females with unrestricted unilateral left-hemisphere damage. This finding was in part explicable on the basis of a higher proportion of vascular accidents in males, causing more extensive pathology within the hemisphere. As well, in the present sample, when vascular accidents caused restricted damage, they more often affected the posterior than the anterior part of the hemisphere. Since speech disorders were found to occur less often in females from posterior than anterior damage, vascular lesions would tend not to affect the critical speech areas in women. Consequently, aphasia would occur less often in females after left-hemisphere damage, and it might appear that their speech was

more bilaterally organized than that of males. There was, however, no suggestion in the large sample of patients with right-hemisphere damage that speech was more often unilaterally represented in the right hemisphere in females than in males. In the light of these findings, one must critically re-examine the question of a more bilateral organization for speech in females.

The only previous direct evidence for some right-hemisphere involvement in speech in females is McGlone's (1980) suggestion of a lowered Verbal IQ in females with right-hemisphere damage, compared with a normal control group. In the present study, females with right-hemisphere damage had a lower Verbal IQ than did males with right-hemisphere damage, although the female scores were near normal for the population. It is of course problematic what the appropriate comparison group should be, and what inferences can be drawn from scores which are impaired relative only to a supranormal standard. It is not impossible, for example, that the difference in Verbal IQ between males and females with right-hemisphere lesions is due to a slightly enhanced verbal intelligence in males after right-hemisphere damage, rather than to reduced verbal intelligence in females.

The lower Verbal IQ scores in women with right-hemisphere lesions in both McGlone's and the present study may thus be taken as only weak support for the idea that the right hemisphere is more involved in verbal function in women than in men. Such findings may have some impact on how one views the representation of higher-level linguistic function in the right hemispheres of males and females, but this may not speak to the issue of how the more fundamental nuts-and-bolts level of speech is organized, since the two aspects of language are not synonymous. It appears that it is particularly the production and perhaps the phonemic-level comprehension of speech which is espe-

cially dependent on the left hemisphere, rather than the symbolic-linguistic aspects of language (Annett, 1973; Kimura, 1979; Zaidel, 1978).

It is not clear what broader implications can be drawn from individual differences in the organization of speech and praxic function along the anterior-posterior dimension. It may well be that both males and females vary more in the neuroanatomical organization of these functions than was previously suspected. However, the dearth of intellectual deficits after posterior lesions in females in the present study is remarkable. Only a negligible number of women with either right or left posterior lesions had any difficulty with constructional or praxic function, in contrast with the severe effects of anterior, especially left anterior, damage. This is not likely to be due to smaller posterior than anterior lesions on the average in females, since there is no medical evidence that this is so in our sample. Moreover, one would have to assume the same effect for the right hemisphere, and to assume the reverse effect if anything, in males, which together comprises a set of improbable assumptions. Furthermore, there are in the present study several cases of males with restricted parietal-lobe damage and a severe apraxia, and several females with at least equal parietal-lobe damage and no apraxia.

The finding of minimal speech and praxis deficits after left posterior lesions in females, and of striking deficits after anterior lesions, must suggest either that these functions are primarily vested in the anterior systems, or that the overlap in function between posterior regions in the female is so great as to approach duplication. The latter hypothesis seems somewhat improbable, particularly in the light of the devastating effect on praxic function produced by left anterior damage. However, one must keep in mind that this was a retrospective study, and that selected data were analyzed for presentation, that is, intelligence test scores and tests of speech and praxic motor function. A wide variety of human abilities is not sampled by either of these sources of data. Basic perceptual functions, face perception, social intelligence, verbal fluency, memory, and a number of other abilities still await intensive study (McGlone, 1980), as does a breakdown of intelligence test scores into subfactors which may be somewhat more sensitive to the effects of CNS damage than are global IQ measures (Matarazzo, 1972). When such studies are completed, we may well find selective effects of left or right posterior damage in females. Thus, there is the possibility that some functions (e.g., linguistic) are more diffusely represented in the female and thus show more duplication between hemispheres (McGlone, 1980), whereas others (e.g., praxic functions) are focally organized.

The importance of the left anterior region in females for the control of speech and praxic functions is, however, unambiguous. Moreover, apart from constructional tasks, these functions do not appear to be shared with the right anterior region, as evidenced by the lack of overlap in scores on manual praxis. If, therefore, there is significant overlap in function between left and right hemispheres in females, it is not apparent with anterior lesions, for either speech or praxic function.

A word of caution is in order. The localization analysis in the present study employed the classification "anterior" and "posterior" because it was relatively easy to define, and because there is some precedent for doing so. It would be naive to assume, however, that the functional systems underlying the abilities we study divide themselves in this arbitrary manner. Moreover, both these regions are very large, and there are of course major functional subdivisions within them which are already known. An exhaustive study with respect to lesion location was not the aim of the present study, except to rule out obvious differences between male and female subgroups in this regard. A more detailed analysis of the lesions will be several years in coming to fruition, because it requires larger sample sizes for both males and females.

One might ask what the benefits of focal organization are for oral and manual praxis. Since such functions are very basic to motor skill (Kimura, 1979), it may be that a more focal organization is advantageous to both the precision and speed which are characteristic of well developed motor skills. Other functions, such as finding one's way, which are less demanding of precise output to the musculature, could with impunity be less focally organized. This of course holds for both men and women. The particular factors which might lead to praxic function being more anteriorly organized in females, while they appear to depend more on parietal regions in males, must at this point be purely speculative. If evolutionary pressures were greater in males for developing the ability to encode certain spatial information (Maccoby & Jacklin, 1974) about the external environment, then there might be some tendency to develop motor skill vis-a-vis the distance receptors, particularly vision. This might account for a "migration" of certain aspects of motor control to posterior regions in males, whereas in females no such evolutionary pressures operated. Alternatively, one might equally well speculate that praxic function in females migrated anteriorly because it was advantageous for fine motor skills, at which females excel, (Tyler, 1965), or advantageous to be coordinated with speech-articulatory function, at which females also excel (Maccoby, 1966). Until we know a great deal more about the functional

subsystems of the brain in both men and women, we cannot possibly answer these questions definitively.

How does one reconcile several normative studies which also suggest more asymmetric functioning of the two hemispheres in males, with the findings of the present study? Most normal studies on lateral asymmetries employ auditory or visual stimuli, and it is reasonable to suppose that these techniques sample the function of regions not too far removed, synaptically, from primary receiving areas. Thus dichotic listening studies may not generally sample asymmetry beyond the superior temporal gyrus or the insula, regions known to receive direct input from auditory cortex; tachistoscopic studies employing visual stimuli may not sample asymmetric functioning much beyond the peristriate region (Kimura & Durnford, 1974). Auditory, visual, or even tactual laterality studies in normal persons probably do not tap the functions anterior to the central fissure which appear to predominate in females. It would follow that such studies would tend to show greater perceptual asymmetry in males than in females. Admittedly, this implies somewhat different neural pathways in males and females for processing sensory input as verbal, but some such difference is an inevitable consequence of a sex difference in neural organization for speech. An additional factor in the reduced perceptual asymmetries in females may be the recently reported larger commissural system in the posterior part of the corpus callosum in females (de Lacoste-Utamsing & Holloway, 1982).

It is therefore of particular interest that if one looks, not at perceptual asymmetry, but at motor asymmetry where posterior systems probably exert less influence than over perceptual processing, the sex difference is, if anything, reversed. Thus, right-hand preference is more prevalent in females than in males (Annett, 1970). Even within right-handers, males do not show a stronger preference for the use of the right hand than do females, and there is no significant difference between males and females in the degree to which hand-use preference is influenced by the verbal or nonverbal nature of the task (Kilbreath, 1979; Kimura, 1973; Hampson & Kimura, Note 1).

A similar problem in interpretation exists for studies on the development of lateralization in the brain. The shifting patterns of lateralization in the course of development and the sex differences in such lateralization, which are sometimes reported in normal children, may well reflect shifts in the involvement of posterior/anterior systems, rather than in overall brain asymmetry. The present study does not refute the idea that there are sex differences in the lateral organization of speech, but it suggests that some elaboration of that general hypothesis is needed. One of the most compelling adjunct hypotheses must be that the intra-

hemispheric organization of speech differs between males and females. A corollary hypothesis is that if there is greater overlap in function between hemispheres in females, such overlap probably involves posterior regions more than anterior.

## Note

1. Hampson, E., & Kimura, D. Hand movement asymmetries during verbal and nonverbal tasks (Department of Psychology Research Bulletin #567). Unpublished manuscript, University of Western Ontario, 1982.

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It is well established that brain size is approximately 10% larger in men than women (e.g., Dekaban and Sadowsky, 1978), but the significance of this disparity is not known. The difference in brain size is likely manifested in a difference in cortical volume, which in turn must be reflected in some sex difference in cellular characteristics, to date undelineated. The total number of cortical neurons depends on the number produced during proliferation and which ultimately survive early cell death (Rakic, 1988). This number might be the same in men and women, which could result in greater numerical density of neurons in women than men and therefore in different neuropil characteristics. Alternatively, factors in sexual differentiation of the brain (McEwen, 1983) could influence neuronal proliferation and survival such that numerical density is similar between the sexes, but the total number of neurons would be less in women than men. The microscopic differences in either scenario could have functional implications. Both models are likely too simplistic. Different laminar and columnar structures of the cortex may be affected by multiple factors operating differently in the sexes.

Sex differences have been documented in specific parts of the brain, such as size of the interhemispheric commissures. Area of the midsagittal section of the posterior region of the body of the corpus callosum (the isthmus) (e.g., Witelson, 1989; Habib et al., 1991; Steinmetz et al., 1992), and of the anterior commissure (Allen and Gorski, 1991; Witelson and Kigar, 1993) was found to be absolutely as well as relatively larger in women than men. In contrast, size of the total corpus callosum, the genu, and the splenium tends to be smaller in women (Witelson, 1989; Allen et al., 1991). In the primate brain, the interhemispheric axons that cross through the isthmus arise from and terminate in neurons in posterior temporal and parietal regions surrounding the posterior part of the Sylvian fissure (Pandya and Seltzer, 1986). These cortical regions also show sex differences in gross morphology in the human brain. The length of the horizontal segment of the posterior Sylvian fissure and the area of surrounding gyri

such as the planum temporale (the superior surface of the posterior part of the superior temporal gyrus within the Sylvian fossa) are greater in both cerebral hemispheres in men than women, although length of other segments of the Sylvian fissure shows no sex difference (Witelson and Kigar, 1991, 1992; Aboitiz et al., 1992).

Sex differences have also been found in the behavioral correlates of parietotemporal regions. Hand preference was found to be associated with size of the isthmus in men, but not in women (Denenberg et al., 1991; Habib et al., 1991; Witelson and Goldsmith, 1991), and with morphology of the posterior Sylvian fissure regions (Witelson and Kigar, 1992). Aphasia may follow left-sided lesions in this region less frequently in women than in men (Kimura, 1987). These results suggest that the functions mediated by parietotemporal regions may not be identical in men and women. Sex differences exist in cognitive functions such as verbal fluency and visuospatial perception (Halpern, 1992) which are mediated by these cortical regions (Hécaen and Albert, 1978). Such findings would be compatible with sex differences in the microscopic structure of parietotemporal cortex. However, little attention has been paid to the sex of the person in studies of the microscopic structure of human cortex. In an influential article, Rockel et al. (1980) concluded that basic uniformity exists in cortex in that the number of neurons in a column through the depth of the cortex is constant across mammalian species, within a species, and even among different cytoarchitectonic areas within the human brain except for striate cortex. However, they studied only two human brains, both male.

This study investigated whether there are differences between men and women in quantitative aspects of the microscopic structure of posterior temporal cortex. We focused our analysis on cytoarchitectonic area  $TA_1$  (von Economo and Koskinas, 1925) which covers the largest surface area of the posterior region of the superior temporal gyrus including the planum temporale, and which has a high degree of individual variability

in its expanse (von Economo and Horn, 1930; Galaburda et al., 1978).

The specific aim of the study was to assess cortical depth and the numerical density of neurons for the full depth of the cortex and for each lamina in this cytoarchitectonic region in male versus female brain specimens. Additionally, since the posterior boundary of area  $TA_1$  and of the planum temporale are not well documented, this study provided the opportunity to map the cytoarchitecture of parts of the superior temporal gyrus not studied previously.

## Materials and Methods

### Brain Specimen Source

The specimens were selected from a brain collection started in 1977, designed for study of the relationship between structure and function in cognitively normal adults. The brains were obtained through autopsy from people with metastatic cancer who were essentially free of adverse signs of the disease when recruited as research subjects and who agreed to participate in a study involving both neuropsychological testing and, in the event of death, an autopsy to allow study of their brain. All subjects were documented to be within the limits of normal variation in cognitive function at the time of testing on the basis of detailed medical and social histories, medical consultations, and neuropsychological test results. Details of the recruitment procedure, neuropsychological testing, and characteristics of the brain collection are described elsewhere (Witelson and McCulloch, 1991).

The entire brain including the medulla was removed by the pathologist during autopsy following a standard procedure. Details are given elsewhere (Witelson and Kigar, 1992). Autopsies were done quickly, frequently within 2–3 hr after death. After removal, the brain was suspended by the basilar artery and fixed in a 10% buffered formalin phosphate solution. Brain weight was measured at autopsy and again 3 weeks after fixation with a Sartorius balance (model U 3600).

### Selection of Brain Specimen Sample

Histologic analysis was done on a sample of nine brains (five women and four men), selected from a total of the 71 brain specimens available at the start of this study. Initially, 10 brains were selected, but one male brain had to be excluded because the required sections in one hemisphere proved to be oblique to the pial surface. Mean time interval between death and brain fixation at autopsy was 7.2 hr for the women and 3.5 hr for the men. All subjects were Caucasian. Aside from sex, an attempt was made to have as homogeneous a sample of brain specimens as possible, and cases were chosen to have the following criteria.

1. All specimens were chosen from people who remained free of neurologic complications or any neuropsychiatric disorders until death.
2. There was no gross or microscopic pathology observed on clinical neuropathologic examination.
3. All specimens had a fixed brain weight that was no more than 1 SD below the mean value typical for that sex and chronologic age based on large groups (Dekaban and Sadowsky, 1978).
4. All specimens were chosen from people who demonstrated on testing consistent-right-hand (CRH) preference, defined as only right-hand preference on a series of 12 items taken from the Annett (1967) handedness questionnaire. Subjects who showed any left-hand preference, even if they used their right hand for writing [classified as not consistent-right-handed (nonCRH)] were not included in this sample. The factor of hand preference was controlled because previous findings showed that the gross morphology of parietotemporal structures was different between CRH and nonCRH men (e.g., Witelson, 1989; Witelson and Kigar, 1992).
5. Specimens were selected to have the more prevalent morphologic pattern of greater expanse of the planum temporale (PT) in the left than right hemisphere (e.g., Geschwind and Levitsky, 1968; Witelson and Kigar, 1988). Details of the definition of PT boundaries are given in the next section.
6. Cases were selected from those who had extensive neuropsychological testing to enable future study of the relationships between microscopic structure and cognitive functions.
7. Among the cases meeting the previous criteria, age at death was selected to make the two groups as comparable as possible.

Table 64.1 gives descriptive data for age, brain weight, and intelligence test scores for each sex group. The stringent criteria we used to select a sample for study has advantages and disadvantages. Although sample size is small, it was anticipated that the homogeneity among the cases might provide sufficient statistical power to detect even small differences that might exist. However, the specificity of the group in anatomical and psychological characteristics limits the generalizability of the results to the general population without additional studies.

### Location of Cytoarchitectonic Area $TA_1$ ; Definition of Planum Temporale (PT)

The tissue blocks for histologic study included cytoarchitectonic area  $TA_1$  (von Economo and Koskinas, 1925), also referred to as area 22 (Brodmann, 1909; Blinkov, 1949) and area Tpt (Galaburda and Sanides, 1980). This area is found on the lateral surface of the posterior region of the superior temporal gyrus accord-

**Table 64.1**

Descriptive data for the male and female groups of brain specimens

	Men ( <i>n</i> = 4)			Women ( <i>n</i> = 5)		
	$\bar{X}$	(SD)	Min–Max	$\bar{X}$	(SD)	Min–Max
Age (years)	48.8 <sup>a</sup>	(16.5)	25–63	53.6	(5.8)	44–59
Brain weight <sup>b</sup>	1425.1 <sup>c</sup>	(47.6)	1384–1494	1246.0	(45.7)	1200–1292
FS IQ <sup>d</sup>	112.3	(14.4)	100–128	110.6	(2.7)	108–115
V IQ <sup>d</sup>	110.0	(12.3)	97–121	111.2	(7.5)	103–123
P IQ <sup>d</sup>	114.0	(17.0)	97–135	108.6	(11.0)	93–122

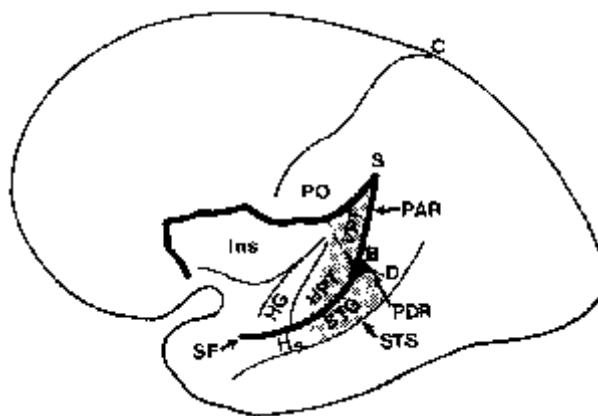
<sup>a</sup>Comparison of sex groups:  $t = 0.65$ ,  $p = 0.54$ .<sup>b</sup>Whole brain weight at 3 week fixation in formalin. These values are similar to those for groups of comparable age in other studies (e.g., 1410 gm for men and 1280 gm for women; Dekaban and Sadowsky, 1978).<sup>c</sup>Comparison of sex groups:  $t = 5.74$ ,  $p = 0.001$ .<sup>d</sup>Full Scale, verbal, and performance IQ scores based on the Wechsler Adult Intelligence Scale (Wechsler, 1955) (comparison of sex groups:  $p > 0.50$  in each case).

ing to the schematic maps in these works and also on the posteriormost region of the superior surface of the supratemporal plane based on series of cytoarchitectonic maps of individual brain specimens (von Economo and Horn, 1930; Blinkov, 1949; Galaburda et al., 1978). The available maps clearly show that no sulcal boundaries exist to demarcate the limits of area  $TA_1$  in a particular brain a priori. Figure 64.1 presents a schematic map of the location of area  $TA_1$ . This representation is a composite of previous maps with a modification based on our data (see Results), showing an extension of area  $TA_1$  onto the vertical wall of the posterior Sylvian fissure.

Area  $TA_1$  was studied in the superior surface of the posterior part of the superior temporal gyrus, that is, in the planum temporale (PT). The posterior boundary of PT has not been definitively established and is particularly ambiguous in some hemispheres. The Sylvian fissure bifurcates in most hemispheres (Witelson and Kigar, 1992) at point B into the posterior ascending and posterior descending rami. On the basis of previous work (Witelson, 1987; Witelson and Kigar, 1992), the posterior border of PT was defined as point S, the end of the ascending ramus. The bifurcation divides PT into horizontal (HPT) and vertical (VPT) components (see figure 64.1). PT has been variously defined: ending at point B (e.g., von Economo and Horn, 1930; Geschwind and Levitsky, 1968), at point D, the end of the descending ramus (Witelson and Pallie, 1973; Steinmetz et al., 1991) or at point S as in this study (also Aboitiz et al., 1992). In hemispheres in which the posterior upward swing of SF is very sharp and anterior in origin (see Witelson and Kigar, 1992, their figure 64.6C), PT has often been considered to be absent (e.g., Wada et al., 1975, their figure 64.1).

#### Brain Dissection and Block Removal

In each hemisphere, the full extent of the Sylvian fossa was exposed by removing the frontal lobe en bloc and

**Figure 64.1**

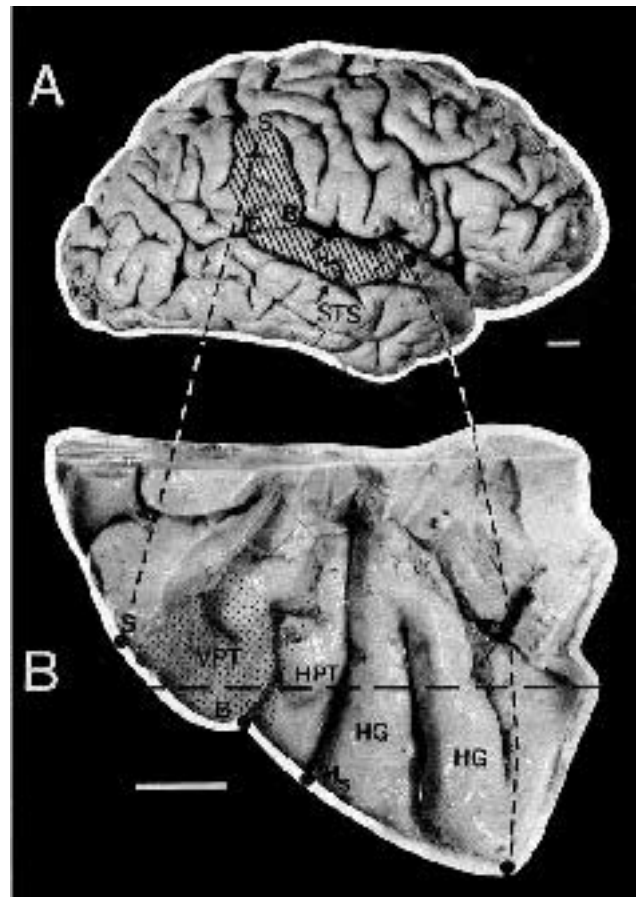
Schematic representation of the gross morphologic features of the planum temporale (PT) and its surround and of the location of cytoarchitectonic area  $TA_1$  based on von Economo and Koskinas (1925) and on our modification. A left hemisphere with typical morphology is shown, with the upper and lower walls of the Sylvian fossa pulled apart (heavy lines represent the lateral edges), exposing the superior surface of the superior temporal gyrus. The Sylvian fissure (SF) bifurcates at point B, into the posterior descending ramus (PDR) ending at D, and the posterior ascending ramus (PAR) ending at S. The floor of the Sylvian fossa exposes Heschl's gyrus (HG) and the two components of the full PT (divided by the dashed line). The horizontal segment (HPT) is in the supratemporal plane, typically considered to be PT, and the vertical segment of the planum temporale (VPT) is on the inner (posterior) wall of the upward curve of PAR. C, Dorsal end of the central sulcus;  $H_s$ , end of Heschl's transverse sulcus on the lateral aspect of the brain; Ins, insula; PO, parietal operculum; STG, superior temporal gyrus; STS, superior temporal sulcus. The localization of area  $TA_1$  is represented by the stippled area. Our modification is the inclusion of  $TA_1$  in the cortical wall in VPT.

then cutting from S, the end of the SF (see figure 64.1), through the parietal and occipital lobes to remove the postcentral part of the parietal lobe including the parietal operculum. This dissection exposed Heschl's transverse gyrus and the full PT in the supratemporal plane and in the upward curve of SE. In this sample of nine brains, 56% of hemispheres had both HPT and VPT components. Three hemispheres (two male, one female) had only a VPT segment (no HPT) because point B, the point of bifurcation of SF, was congruent with Heschl's sulcus, an anatomic variant described elsewhere (Witelson and Kigar, 1992, their figure 64.6C).

A large block of the superior temporal gyrus was removed from each hemisphere. The block included the full superior surface of Heschl's gyrus and the full PT extending all the way to S, thus including both HPT and VPT (Heschl/PT block). The block was bounded inferiorly by the superior temporal sulcus (see figure 64.2). If SF curved sharply at point B, it was sometimes necessary to cut the Heschl/PT block into two parts at point B and section them separately. The sample of area  $TA_1$  used for quantitative measurement was taken in most cases from HPT since it tended to have a greater expanse of area  $TA_1$  with a flat surface suitable for cell counts in sections cut perpendicular to the pia.

Photographs of the Heschl/PT block were taken to measure surface areas for use in obtaining estimates of the surface area of  $TA_1$  and subsequently estimates of cortical volume and the total number of neurons in area  $TA_1$ . For each hemisphere, three photographs were taken, of HPT, VPT, and the lateral aspect of the block. HPT and VPT were photographed separately since the planes of the two segments are almost always different. The lateral area of  $TA_1$  was defined as the region between the extensions of Heschl's sulcus and of point B to the superior temporal sulcus (see figure 64.1). All photographs were made at  $1\times$  magnification based on two orthogonal rulers in the photograph. Area measurements were made using the Bioquant II Digitizing Morphometry Program A5-IA2.

The sum of these three areas was used as the surface area of  $TA_1$ . Since the boundaries of area  $TA_1$  do not necessarily coincide with sulci, this definition of the extent of  $TA_1$  is a very rough approximation. The estimate necessarily includes part of cytoarchitectonic area TB in HPT; the estimate does not capture the undulations of the surface of PT, nor the cortical surface within small sulci, such as the posterior descending sulcus (see figures 64.1, 64.2), and the lateral measure uses gross anatomical features to define an unknown extent of the cytoarchitectonic area.



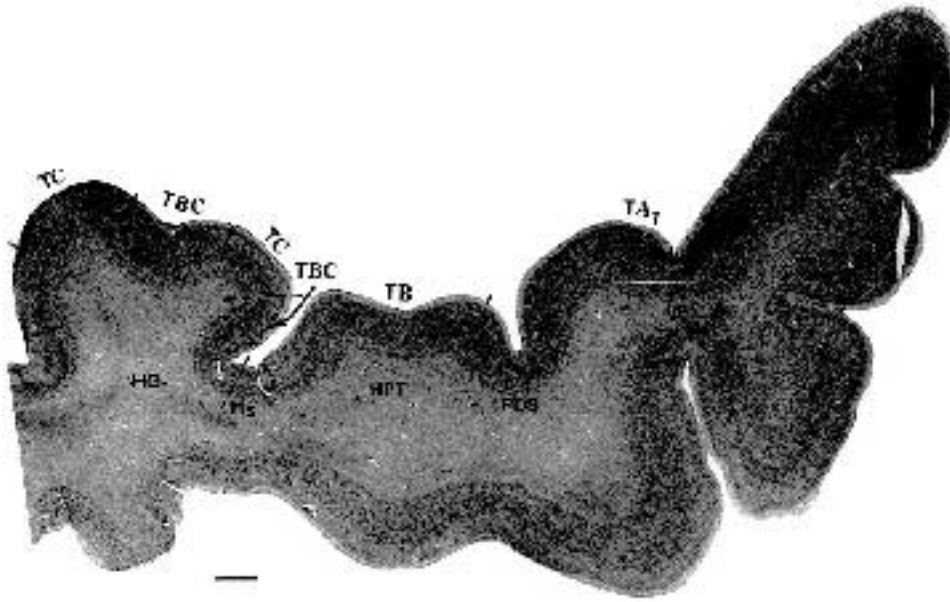
**Figure 64.2**

Photographs of the lateral view of a right hemisphere (A) and the superior view (B) of the Heschl/planum temporale (HPT) block removed from that hemisphere. In this hemisphere, the horizontal and vertical components of PT could be removed in the same block. This photograph is in the plane of HPT; VPT is foreshortened. The brain was cut through PAR to reveal VPT. Point B indicates where PDR meets the lateral edge. It can be seen that PDR does not extend as far medially as does PAR. The *dashed lines* connect anterior and posterior points in the H/PT block to their position in the hemisphere. The lateral aspect of the whole block is indicated by *hatched lines* in A. The plane of cut of the Nissl-stained sections (*heavy dashed line*) is indicated. The general location of cytoarchitectonic area  $TA_1$  in PT is indicated by the stippled region. Abbreviations are as in figure 64.1. Scale bars, 1 cm.

#### Histological Sectioning and Staining

Since morphology varies among hemispheres, the plane of cut for each block was chosen individually in an attempt to have sections made through a flat posterior PT region and angled perpendicularly to the pia in that region. Generally, the Heschl/PT block was sectioned in a plane perpendicular to the longitudinal axis of Heschl's transverse sulcus (see figure 64.2).

The whole block was frozen with dry ice and cut on a sliding microtome (American Optical) at a constant setting to yield final stained sections with a mean thickness of 25  $\mu\text{m}$ . Pilot work had revealed that sections



**Figure 64.3**

Photograph of a Nissl-stained section through a Heschl/planum temporale (HPT) block showing the location of the four major cytoarchitectonic regions, labeled as in von Economo and Koskinas (1925). The section is from the H/PT block shown in figure 64.2. TC is primary auditory koniocortex and occurs in islands; TBC is a transitional form, both found mainly in Heschl's gyrus (HG); TB is association cortex which covers the anterior region of the PT posterior to Heschl's sulcus ( $H_s$ );  $TA_1$  is association cortex found in the posterior regions of PT (in HPT and VPT). See Results for a description of the cytoarchitectonic characteristics of area  $TA_1$ . Abbreviations are as in figure 64.1. Scale bar, 2 mm.

this thick were needed for recognition of cytoarchitectonic patterns. Every consecutive tenth section was saved and mounted. The orientation of each section in respect to its position in the brain was recorded. The sections were Nissl-stained with 0.1% cresyl violet solution. These sections were used to identify area  $TA_1$  and for quantitative analysis. Mean linear shrinkage of the sections through the depth of the cortex was 8.1%, based on assessment of seven tissue blocks from four brains.

#### Cytoarchitectonic Analysis

To ensure our ability to distinguish among the different cytoarchitectonic areas in the region of PT, and specifically to identify area  $TA_1$  as described by von Economo and Koskinas (1925), we studied all the serial sections of Heschl/PT blocks, including the HPT and VPT components, prepared as described in previous sections, for three brains (six hemispheres). This included approximately 80–100 sections per hemisphere and cytoarchitectonic mapping was done for the full extent of each slide.

We were able to distinguish four main cytoarchitectonic regions comparable to von Economo's areas TC, TBC, TB, and  $TA_1$ . They occurred always in the same topographic progression in a general anterior to posterior direction over the Heschl and PT gyri. Figure 64.3 presents a photograph of a typical section of a Heschl/

PT block showing the location of the four major cytoarchitectonic areas. We found, as shown in von Economo and Horn's (1930) detailed maps, that area TC, primary koniocortex, which occurs in islands of tissue, and area TBC, a transitional form, are typically located within Heschl's gyrus; area TB covers the anterior portion of HPT; and area  $TA_1$  is located posterior to area TB in HPT and also in VPT when present.

Differentiation of these areas was based on visual qualitative cytoarchitectonic characteristics such as laminar distribution of cells, cell size, packing density, columnar arrangement, and relative thickness of different layers. Using these criteria, area  $TA_1$  was identified in sections from each hemisphere of the nine brains studied. Identification was done for each brain independently by three raters. In each case, there was 100% agreement as to which cortical region was area  $TA_1$ .

#### Quantitative Analyses

The quantitative analyses used in this study were based on a computer-assisted manual method involving cell differentiation in Nissl-stained sections under the microscope, marking neurons having nucleoli with the aid of a camera lucida, and defining laminar depths using photomicrography. The strict criterion of counting only neurons with nucleoli and the use of thick sections in which most neurons could be seen in three dimensions to aid differentiation of small neurons versus glial

cells help obtain accurate estimates of neuron counts. This direct method, although time consuming, was chosen rather than automatic methods based on computer detection of items according to size (e.g., Terry et al., 1987) because the latter methods likely have more measurement error which could obscure the size of the individual differences that might exist and were being investigated.

The cortical sites of area  $TA_1$  used for measurement were selected so that each site was not at the crest or valley of a gyrus, the pial surface was flat, the cell columns were perpendicular to the cortical surface, and the least amount of technical artifacts and blood vessels were present. Six slides per hemisphere were selected for measurement. In each slide, area  $TA_1$  was identified by three raters, and two adjacent traverses, each 125  $\mu\text{m}$  wide, were analyzed. The sample size of 12 traverses per hemisphere was selected because pilot work had indicated that the variation in cell counts within a hemisphere was not reduced with a number of traverses greater than twelve.

**Cortical and Laminar Depth** Low power photomicrographs of the sites chosen for measurement for each slide were used to delineate the six cortical layers. The common cytoarchitectonic features such as packing density of the cells and their size and arrangement in cytoarchitectonic columns were used for defining the boundaries between the cortical layers. The boundary between the cortex and subcortical white matter was defined by the presence of no more than three neurons in the last counting field. For each photomicrograph, there was a paired photograph of the stage micrometer taken at the same magnification, which was used as a ruler to measure the depth of each layer. Using the magnification factor, laminar divisions were indicated on the camera lucida drawings to calculate the number of neurons in each lamina.

**Neuronal Counts** Neurons were counted in two adjacent traverses from the pia to the edge of the white matter. Neurons were differentiated from glia under high magnification (630 $\times$ ), using typical features such as general size of the cell, size and structure of the nucleus, and distribution of the nuclear chromatin. Those neurons having nucleoli were counted by marking them via a camera lucida onto sheets, each sheet representing one microscopic field. Errors in counting (double counting or omission) were kept in check with the aid of an eyepiece grid (10 mm by 10 mm) that was used to outline consecutive microscopic fields through the depth of the cortex. The frame of the grid defined the microscopic field of 125 by 125  $\mu\text{m}$  on the slide as seen at the magnification used. The neurons within the grid and those which crossed the top and right-side

borders of the frame were counted. The region of cell counting could be found again by marks made directly on the slide and by tracings of artifacts made on the camera lucida drawings. This allowed ambiguous cells to be checked with a second investigator. The number of neurons was counted for each layer from the tracings with the aid of a computer. All cell counts were corrected using an Abercrombie correction to adjust for the varying thickness among sections. Intrajudge reliability revealed counts within 1–2% for the same traverse done on different days. Interjudge reliability was checked in a few cases and was found to be within 2–5%.

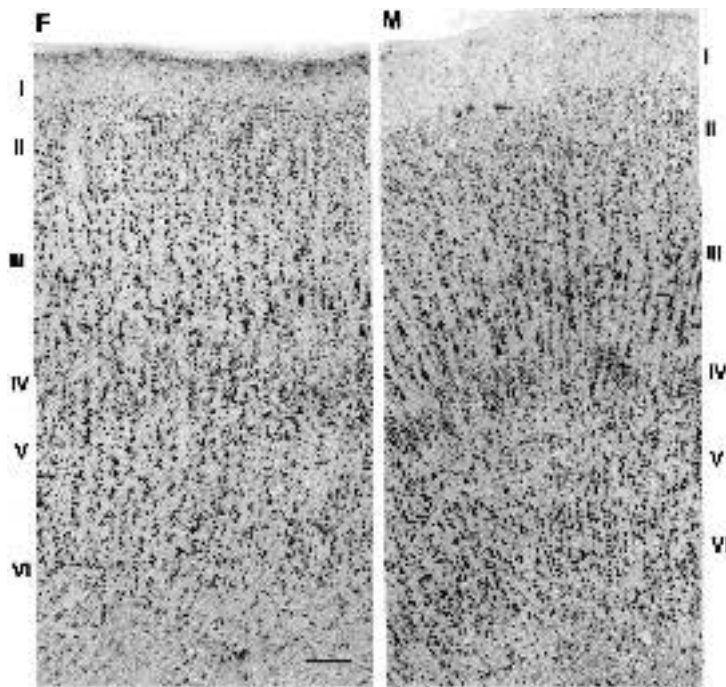
**Section Thickness** Numerical densities were calculated from the raw count numbers for each slide by using the measured section thickness for that slide. Although the frozen tissue was cut at a constant setting, section thickness varied among the slides. Thickness was measured at six different sites through the depth of cortex for each slide. This was done under oil immersion by focusing from the top to bottom of the section and reading the values from the fine focus knob of the microscope. Section thickness was calculated based on the focus knob reading and the refractive index of oil (1.515). For each slide, the mean value of the six measures was obtained. Mean section thickness for all slides was 24.9  $\mu\text{m}$  (min/max = 17.0/34.6).

**Histologic Variables** The following measures were obtained for each slide.

1. Cortical depth, depth throughout the cortex (total depth) and depth of each cortical lamina (micrometers): this variable was obtained by direct measurement and for each slide is the mean value for the two traverses.
2.  $N_v$ , number of neurons under 1  $\text{mm}^2$  of cortical surface through the total depth of the cortex, and through the depth of each layer: this is columnar neuronal number, that is, an estimate of the number of cells in a column perpendicular to the pial surface with volume equal to 1  $\text{mm}^2 \times d$  mm, where  $d$  is either total cortical depth or laminar depth.

This variable was calculated for each slide according to the equation  $N_v = n/(w * t)$ , where  $N_v$  is the number of neurons under 1  $\text{mm}^2$  of cortical surface,  $n$  is the mean number of neurons from two traverses,  $w$  is the width of the traverse (in mm), and  $t$  is the mean section thickness (in mm).

3.  $N_v$ , number of neurons per unit volume (1  $\text{mm}^3$ ) for total cortical depth and per unit volume for each layer (often called cell packing density): for total cortical depth,  $N_v$  is the value for the total column, not the mean of the six laminar values for  $N_v$ .



**Figure 64.4**

Photomicrographs of Nissl-stained sections showing cytoarchitectonic area  $TA_1$  (described in Results) in the posterior region of the planum temporale in the superior temporal gyrus in one female (*F*) and one male (*M*) brain. Each micrograph represents a section through the full depth of the cortex cut perpendicular to the pia and the six cortical layers are indicated. The width of the transverse for counting was 250  $\mu\text{m}$ . No sex differences were evident by qualitative observation. Scale bar, 200  $\mu\text{m}$ .

This variable was calculated for each slide according to the equation  $N_v = n/(d * w * t)$ , where  $N_v$  is the number of neurons per 1  $\text{mm}^3$  of cortical tissue,  $n$  is the mean number of neurons from two traverses,  $d$  is the mean cortical or laminar depth of the two traverses (in mm),  $w$  is the width of the traverse (in mm), and  $t$  is the mean section thickness (in mm). Abercrombie corrections were applied here.

For each slide, the depth and numerical density values, in combination with the estimates of  $TA_1$  surface area, were used to calculate estimates of the cortical volume of area  $TA_1$  and the total number of neurons in  $TA_1$  and in each layer of  $TA_1$ . For each hemisphere, the value for each variable was the mean of the scores from the six slides. The final score used in the statistical analyses was the mean of the two hemisphere scores (see next section).

The mean value for  $N_e$  obtained for the sample of four male brains in this study is almost identical to that reported by Rockel et al. (1980) for two brains using a similar method (see Witelson et al., 1992). The mean values obtained for  $N_v$  were well within the range of  $N_v$  values reported for various cortical regions in recent studies using the optical dissector method (Braendgaard et al., 1990; Pakkenberg, 1993). [A few older studies using manual methods reported  $N_e$

values, but often only on single cases, with sex unspecified, or extent of tissue shrinkage not indicated (see Blinkov and Glezer, 1968).] These comparisons provide some external validation for the methods and representativeness of the sample in the present study.

#### Statistical Procedures

Descriptive statistics were obtained for all variables. Two-way analyses of variance (ANOVA) were done for each variable with Sex and Hemisphere as between factors. There was no interaction between factors; in other words, the sex differences were the same for each hemisphere. This report addresses the factor of Sex. Accordingly, all scores represent the mean of the scores for the two hemispheres of each brain. The factor of Hemisphere itself will be the subject of a subsequent report. All percent difference scores were calculated using the mean of the two scores for the denominator  $[(F - M)/((F + M)/2)] \times 100$ , where  $F$  = female and  $M$  = male. Independent two-tailed Student's  $t$  tests and Pearson product-moment correlations were used with significance level set at  $\alpha = 0.05$ . Differences of at least 10% were considered biologically important and were reported as a tendency even if they were not statistically significant with the present small sample size.

**Table 64.2**Means<sup>a</sup> and SDs for cortical depth,  $N_c$ , and  $N_v$  for the samples of five women and four men

Layers	Cortical Depth ( $\mu\text{m}$ )		$N_c$ (in Thousands)		$N_v$ (in Thousands)	
	Women $\bar{X}$ (SD)	Men $\bar{X}$ (SD)	Women $\bar{X}$ (SD)	Men $\bar{X}$ (SD)	Women $\bar{X}$ (SD)	Men $\bar{X}$ (SD)
I	276 (26)	293 (51)	4 (1)	4 (2)	13 (4)	13 (3)
II	275 (30)	277 (19)	25 (3)	22 (4)	91 (2)	81 (12)*
III	810 (117)	902 (104)	38 (5)	39 (8)	47 (4)	44 (6)
IV	320 (41)	316 (25)	27 (4)	23 (5)	83 (7)	68 (9)**
V	433 (76)	469 (142)	19 (3)	20 (7)	45 (2)	42 (3)
VI	622 (70)	666 (79)	18 (2)	18 (2)	27 (4)	25 (4)
Total	2735 (156)	2923 (341)	130 (7)	125 (16)	48 (2)	43 (1)***

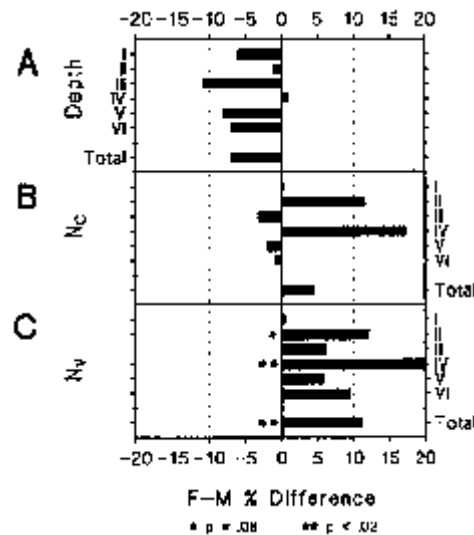
 $N_c$  is the number of neurons under  $1 \text{ mm}^2$  of cortical surface;  $N_v$  is the number of neurons per  $\text{mm}^3$  of tissue.<sup>a</sup>Scores are the means of the right and left hemisphere values.\*,  $p = 0.08$ .\*\*,  $p = 0.02$ .\*\*\*,  $p = 0.008$ , for independent  $t$  tests,  $df = 7$ .

## Results

### Cytoarchitectonic Analysis

Area  $TA_1$  was found in the posterior region of PT in each hemisphere and corresponded to the pictorial and verbal description of other authors (e.g., von Economo and Koskinas, 1925). As reported in previous studies (von Economo and Horn, 1930; Galaburda et al., 1978), there was much individual variation in the location and extent of area  $TA_1$ . Cortical tissue was analyzed in the horizontal supratemporal surface (HPT) and in the surface of the posterior wall of the Sylvian fossa when it curves upwards within the posterior ascending branch of SF (VPT) (see figure 64.1). It was found, as documented previously, that area  $TA_1$  is located in the posteriormost region of HPT.  $TA_1$  cortex was found also in VPT (see figure 64.1). Of particular relevance for cytoarchitectonic mapping,  $TA_1$  tissue was evident in VPT cortex in each of the three hemispheres in which there was no HPT.

Area  $TA_1$  can be recognized by the following characteristic cytoarchitectonic features. Radial striation is well expressed and in most cases radiation extends from the upper part of layer III into layer VI. However, these cytoarchitectonic columns are less regular than in area  $TB$ , and the radial striation in layers V and VI is less evident than in area  $TB$ . The border between layers II and III is much less distinct than in area  $TB$ . Layer III is composed of wide columns (about 3–4 cells across) of pyramidal cells of a progressively larger size ( $50 \mu\text{m}$  or more) until the border at which there is intermingling with granular cells of layer IV. The cells in layer V are larger than in layer VI, but their density is less than in layer VI and than in layer V of area  $TB$ . Figure 64.4 presents photomicrographs of a sample of area  $TA_1$  from one female and one male brain. No sex differences were evident by qualitative observation.

**Figure 64.5**

Bar graphs depicting the percent difference between female ( $F$ ) and male ( $M$ ) brains for cortical depth ( $A$ ),  $N_c$  ( $B$ ), and  $N_v$  ( $C$ ) for total cortex and for each layer for area  $TA_1$ . Scores are means of right and left hemispheres. The percent difference is  $[(F - M) / ((F + M) / 2)] \times 100$ .  $N_c$  is the number of neurons under  $1 \text{ mm}^2$  of cortical surface.  $N_v$  is the number of neurons per  $1 \text{ mm}^3$  of tissue. The dotted lines highlight differences of at least 10%.

Table 64.2 presents means, SDs, and statistical test results for the three variables (depth,  $N_c$ ,  $N_v$ ) for the total cortex and for each layer for each sex group.

### Cortical Depth

As shown in table 64.2, cortical depth did not differ between the sexes for either total cortex or for any layer. Figure 64.5A shows the percent difference between the sexes for cortical depth. There was a tendency for layer III to be greater in men than women by 11%, and a suggestion of a similar tendency for layers I, V, and

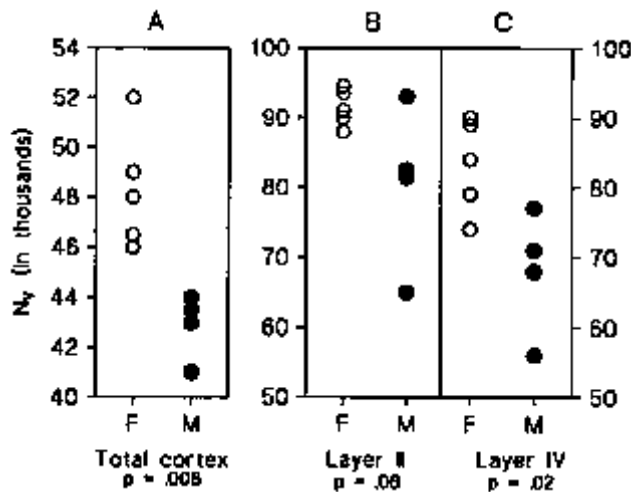


Figure 64.6

Plot of values for  $N_v$  in area  $TA_1$  for each of the five female (F) (open circles) and four male (M) (solid circles) brains studied. Scores are means of right and left hemispheres. A, Total cortex; B, layer II; C, layer IV.  $N_v$  is the number of neurons per 1 mm<sup>3</sup> of tissue (in thousands).

VI. In contrast, layers II and IV showed virtually no difference between the sexes.

#### $N_c$ , Number of Neurons under 1 mm<sup>2</sup> of Surface through the Depth of Cortex (Columnar Neuronal Number)

As shown in table 64.2,  $N_c$  did not differ statistically between men and women for total cortex or for any layer. Figure 64.5B shows the percent difference between the sexes.  $N_c$  tended to be greater in women by approximately 12% in layer II and by 17% in layer IV.  $N_c$  for the other layers was very similar in both sexes.

#### $N_v$ , Number of Neurons per Unit Volume (Cell Packing Density)

As shown in table 64.2,  $N_v$  was greater in women than men for the total cortex ( $p = 0.008$ ) and for layer IV ( $p = 0.02$ ). There was a tendency for layer II to be greater in women ( $p = 0.08$ ). Figure 64.5C shows the percent difference between the sexes for each layer.  $N_v$  was greater in women by 11% for the total cortex, by 13% in layer II and by 20% for layer IV.

The quantitative analyses revealed a similarity in values for  $N_v$  between layers II and IV, and between layers III and V (see table 64.2). This was so in each sex.  $N_v$  for the total cortex did not show an association with chronological age in our samples ( $r = -0.15$  for women,  $r = -0.05$  for men).

Figure 64.6 presents the plots of the values of  $N_v$  for each brain for the total cortex, for layer II and for layer IV. There was no overlap between the sexes in  $N_v$  for total cortex (figure 64.6A). In the case of layers II and IV, only one male brain had a value within the interval observed for the female brains (figure 64.6B,C).

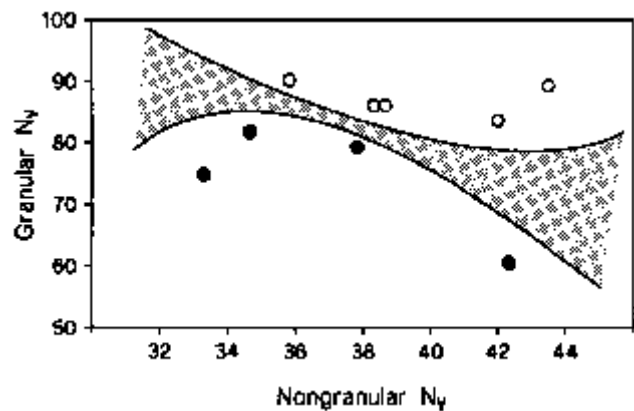


Figure 64.7

Scatterplot of  $N_v$  for granular versus nongranular systems for each female (open circles) and each male (solid circles) brain. The granular values are the means of layers II and IV, the nongranular values are the means of layers III, V, and VI. Scores are means of values for the right and left hemispheres. The stippled area highlights the separation between the sexes. There is no overlap between the sexes for the granular system, in contrast to the almost complete overlap for the nongranular system.  $N_v$  is the number of neurons per 1 mm<sup>3</sup> of tissue (in thousands).

One man was considerably younger (25 years) than all other cases. He was one of the middle data points in the male group in figure 64.6, A and B, and the highest value in figure 64.6C.

#### Granular versus Nongranular Layers

The sex differences in  $N_v$  occurred in layers II and IV (referred to as the granular system), and not in layers III, V, and VI (referred to as the nongranular system). Based on this general pattern, we investigated the relationship between these two parts of the cortical plate (layer I was excluded from this analysis). The mean value of  $N_v$  for layers II and IV was plotted against the mean value of  $N_v$  for layers III, V, and VI for each brain (figure 64.7). There was a discrete separation between the sexes, as highlighted by the stippled region within the scatterplot. The graph shows the lack of overlap between the sexes for granular system  $N_v$  represented on the y-axis, in contrast to the almost complete overlap of male and female scores for nongranular system  $N_v$  represented on the x-axis. The straight-line relationship between the granular and nongranular values for  $N_v$  for men was  $r = -0.78$  ( $p = 0.22$ ), and for women was  $r = -0.29$  ( $p = 0.64$ ). The stippled region within the scatterplot highlights the possibility of curvilinear relationships.

#### Estimated Total Number of Neurons in Area $TA_1$

The total number of neurons in area  $TA_1$  was estimated by the formula  $N_T = a * d * N_v$ , where  $N_T$  is the total number of neurons in area  $TA_1$ ,  $a$  is the estimated total surface area of area  $TA_1$ , defined as the

**Table 64.3**Means<sup>a</sup> and SDs for total number of neurons ( $N_T$ ) in the estimated cortical volume of area  $TA_1$  for total cortex and for each layer for each sex

Layers	$N_T$ (in millions) <sup>b</sup>		$F - M\%$ Difference <sup>c</sup>	Each Layer as % of Total $N_T$	
	Female $\bar{X}$ (SD)	Male $\bar{X}$ (SD)		Female $\bar{X}$	Male $\bar{X}$
I	3 (1)	4 (2)	-29.6	2.5	3.0
II	23 (8)	25 (13)	-8.3	19.2	18.5
III	36 (16)	44 (16)	-20.0	30.0	32.6
IV	24 (7)	23 (9)	4.3	20.0	17.0
V	18 (8)	21 (8)	-15.4	15.0	15.6
VI	16 (7)	18 (6)	-11.8	13.3	13.3
Total	120 (44)	135 (46)	-10.9	100.0	100.0

<sup>a</sup>Scores are the means of the right and left hemisphere values.<sup>b</sup>For all comparisons, sample size was five women and four men; for each  $t$  test,  $df = 7$  and  $p > 0.35$ .<sup>c</sup> $[(F - M)/(F + M)/2] \times 100$ .

sum of areas of the two components of PT (HPT, VPT) and of the lateral expanse of the posterior part of the superior temporal gyrus (as defined in Materials and Methods),  $d$  is the total cortical depth, and  $N_v$  is the numerical density of neurons per unit volume (as defined in Materials and Methods). The mean estimated surface area of  $TA_1$  was 1086 mm<sup>2</sup> and 916 mm<sup>2</sup> in men and women, respectively (17% difference,  $t = 0.76$ ;  $p = 0.47$ ). The mean estimated cortical volume of  $TA_1$  was 3146 mm<sup>3</sup> and 2513 mm<sup>3</sup> for men and women, respectively (22% difference,  $t = 1.02$ ;  $p = 0.34$ ).

Table 64.3 presents  $N_T$  for the total cortex and for each layer for each sex. Percent sex differences are also given. No differences reached statistical significance, but  $N_T$  in each of the nongranular layers (III, V, and VI) tended to be greater in men than women by about 12% or more. In each granular layer,  $N_T$  was very similar between men and women. Table 64.3 also gives  $N_T$  per layer as a percent of  $N_T$  for the total area  $TA_1$  cortex.

## Discussion

### Sex Differences in Cell Packing Density

Cell packing density through the full depth of the cortex in cytoarchitectonic area  $TA_1$  in both hemispheres was found to be 11% greater in the brains of five women compared to four men, all documented to be cognitively normal and consistently right-handed. There was no overlap in  $N_v$  between the sexes (see figure 64.6A, Results). To our knowledge this is the first report of a sexual dimorphism in a quantitative microscopic feature of human cortex. These results provide a first step to delineating a cellular basis that might underlie the sex difference in overall brain size, a difference which is well documented but whose significance is not known. The sex difference in  $N_v$  may reflect dif-

ferences in connectivity and synaptology which could have physiological and behavioral consequences.

Neither total cortical depth nor the number of neurons in a column of cortex under 1 mm<sup>2</sup> of cortical surface ( $N_c$  was different between men and women. The similarity in  $N_c$  is consistent with the hypothesis of uniformity of cortex for  $N_e$  (Rockel et al., 1980).

The results in this study appear clear, but they are based on a small sample and need replication in other samples to gain greater confidence. Additionally, because the sample studied was selected to be homogeneous in several specific characteristics, such as hand preference, caution must be taken about generalizing the results to the entire population before broader samples are investigated. In this regard, our preliminary results with a sample of six left-handers are relevant as they also indicate greater  $N_v$  in women than men. It also remains to be determined whether the observed sex difference extends to cortex in other regions of the brain.

Some considerations support confidence in these findings. The variation of  $N_v$  scores within each sex was small, as evidenced by the low SDs (2–4% of the mean scores), suggesting that the mean scores may be good estimates of the true numerical densities for each sex. The fact that the  $N_v$  difference occurred only in one type (granular) of layer suggests that the finding is not due to a methodological artefact. The mean  $N_v$  values we obtained of 48,000 and 43,000 (see table 64.2) for women and men, respectively, are quite comparable to  $N_v$  values obtained with larger samples in studies using the optical disector method. Braendgaard et al. (1990) calculated a mean value for  $N_v$  for temporal cortex (cytoarchitectonic region not specified) of approximately 46,000 in a sample of five 80 year old male brains (min/max = 38,000/53,000). Pakkenberg (1993) reported a mean value of  $N_v = 40,400$  for temporal cortex for a group of 16 men having a mean

age of 60 years. In a preliminary study involving 18 men and 8 women, Pakkenberg et al. (1989) reported only one mean value ( $N_v = 45,000$ ) for the overall cortex, including occipital cortex. They stated no variation was observed with age or sex but no values were reported.

While the results reported here must be verified in other studies before they can be generally accepted, the following comments are offered. The magnitude of 11% for the sex difference in  $N_v$  for the total cortex corresponds closely to the 10% sex difference in brain size (Dekaban and Sadowsky, 1978). Such a result raises the hypothesis that one possible cause of the greater  $N_v$  in women is a simple mechanical compression or geometric consequence of a smaller brain. The sex difference in brain size has generally been argued to have little biological significance on the basis of no sex differences in general cognitive abilities such as overall intelligence or memory (Halpern, 1992) and similar ratios of brain size to measures of body size in men and women (Gould, 1981). However, the assumption of an allometric relationship is undermined by the very low correlations between brain and body size variables within male and female groups (Peters, 1991), and also by the different pattern of sex differences observed for different cortical layers (discussed in a subsequent section). Cortical metabolic rate (expressed per unit volume) has been found to be greater in women than men in some PET studies (e.g., Hatazawa et al., 1987). Our results suggest that the greater  $N_v$  in female cortex may contribute to this metabolic difference.

#### Granular versus Nongranular Systems

The laminar analyses revealed a disparity in the pattern of similarities and differences between the sexes for layers II and IV (the granular system) and layers III, V, and VI (the nongranular system). Our small sample size likely provided insufficient power to detect all the laminar differences that may exist. Differences of at least 10% which did not reach statistical significance but which might be of biological importance are discussed as tendencies. Figure 64.8 presents a summary of the sex differences in the two systems. This set of findings requires a multifactorial model of the origin of sex differences in cortical microscopic structure. In the granular system,  $N_v$  was greater in women (by 17%), resulting from similar cortical depth and a tendency (15%) for greater  $N_c$  compared to men. The disparity in  $N_v$  between women and men suggests different connectivity in granular layers. The combination of the tendency for area  $TA_1$  to be greater in men (17%) and their lower  $N_v$  resulted in a similar estimated total number of neurons in layers II and IV in men and women. The pattern of sex differences in the granular system fits with the compression model.

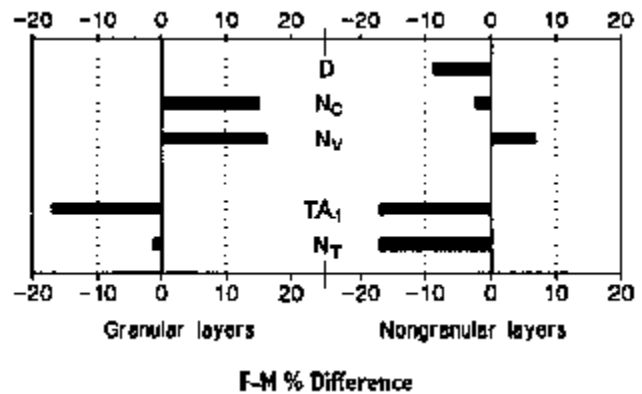


Figure 64.8

Summary chart showing percent difference between male ( $M$ ) and female ( $F$ ) brains in cortical depth ( $D$ ),  $N_c$ ,  $N_v$ , estimated total surface area of region  $TA_1$  and estimated total number of neurons ( $N_T$ ) in the cortical volume of area  $TA_1$  for the granular (layers II and IV) and nongranular systems (layers III, V, and VI). Scores are means of right and left hemispheres. The percent difference score is  $[(F - M) / ((F + M) / 2)] \times 100$ . The dotted lines highlight differences of at least 10%.

In the nongranular system, there was no evidence of reliable sex differences in cortical depth,  $N_c$  or  $N_v$ . The larger area  $TA_1$  in men combined with a similar  $N_v$  to women resulted in a tendency for the estimated total number of neurons to be greater in men (17%). This pattern suggests that factors other than those operative in the granular system are in effect here. The difference in the cytoarchitectonic characteristics of area  $TA_1$  between men and women is highlighted by the different correlations between granular  $N_v$  and nongranular  $N_v$  in men ( $r = -0.78$ ) and in women ( $r = -0.29$ ) (see figure 64.7, Results).

Interestingly, in a study of sex differences in cell packing density in the rat, Reid and Juraska (1992) showed that the total number of neurons was greater in the male than female brain in binocular primary visual cortex in all layers except layer IV. Although the authors emphasized the sex differences that were found, of particular relevance to the present result is that only in layer IV, the rat's granular layer, was depth not greater in males and did  $N_v$  tend to be larger in females, resulting in similar total neuron number. The general point is that although these studies deal with very different species and cytoarchitectonic areas, sex differences in neuronal density may be specific to granular layers across mammalian species.

#### Differential Sexual Differentiation of the Cortical Layers

The presence of sex differences in the microscopic structure of  $TA_1$  cortex suggests that some sex-related genetic or epigenetic factors play a role in their development. For  $TA_1$  cortex, as in the case of other organs which undergo sexual differentiation, female cortex may be prototypic (i.e., determined predominantly

by genetic factors) whereas male cortex would be a derivative resulting from the operation of additional biologic factors, such as sex steroids. The presence of different patterns of sex differences and similarities in the laminar systems (granular vs nongranular) suggests that sexual differentiation of the cortex must be different in some respect for the different layers. In any hypothetical model, some factor may result in the greater total number of nongranular cells (by greater proliferation or less cell death) in men than women which, in combination with the greater area of the cytoarchitectonic region in the male brain, results in similar  $N_v$  values. In the granular system the process must work differently, resulting, possibly by default, in similar total numbers of cells which, when packed into the smaller region in the female brain, leads to the greater  $N_v$  in women.

Tracing and immunocytochemical studies indicate that layers II and IV compared to layers III, V, and VI have neuronal populations which have different functions in terms of input and output and which synthesize different transmitters (Naegle and Barnstable, 1989). The granular system is predominantly an input component of the cortical column and contains mostly GABAergic neurons. The nongranular system is mainly an output system with most neurons being glutamate positive. Our results suggest that the cortical functional unit is constituted differently in each sex and that at least for auditory association cortex, men and women who are right-handed differ in the histologic nature of the input layers and in the proportional amounts of the input versus output systems or of the GABAergic versus glutamate systems. This suggests greater thalamocortical flow in women than men. It is not difficult to imagine that such differences could influence receptive fields and have consequences for specific psychologic skills such as aspects of stimulus discrimination or perceptual integration. Delineation of the neuroanatomic substrate of cognition may be furthered by studies which examine the possible cognitive correlates of histologic variables which show individual differences.

#### Expanded Cytoarchitectonic Map of Area TA<sub>1</sub>

Cytoarchitectonic area TA<sub>1</sub>, was reliably identified in each hemisphere, in either the horizontal, vertical, or both walls of the posterior region of the superior temporal gyrus in the Sylvian fissure (see figure 64.1). Our finding that the same cytoarchitectonic region is present in the vertical as in the horizontal surface of the posterior Sylvian fissure supports the conclusion based on gross anatomy that the planum temporale includes the vertical segment in the posterior ascending ramus of the Sylvian fissure (e.g., Rubens, 1976; Witelson and Kigar, 1992). This vertically oriented cortex has not

been mapped cytoarchitectonically before. Von Economo and Horn (1930) analyzed only the horizontal supratemporal cortex. Blinkov (1949) and Galaburda et al. (1978) used coronal sections which often are oblique or even parallel to the vertical wall. In the latter report, study of the coronal sketches suggests that TA<sub>1</sub> type tissue (referred to as Tpt tissue) was sometimes present in cortex on the lateral aspect of the brain adjacent to the vertical upswing of the Sylvian fissure, which buttresses our finding. Definition of the planum temporale including both horizontal and vertical components revealed right-left symmetry in overall size of this region (Witelson and Kigar, 1991, 1992; Loftus et al., 1993), in contrast to the numerous observations of a greater planum temporale on the left side when only the horizontal plane was considered. Clarification and definition of the gross anatomy of this region is important, since currently various definitions of the gross anatomy of this gyral surface are being used in the increasing number of brain imaging studies relating anatomical and psychological measures in normal (e.g., Steinmetz et al., 1991) and clinical groups (e.g., Leonard et al., 1993).

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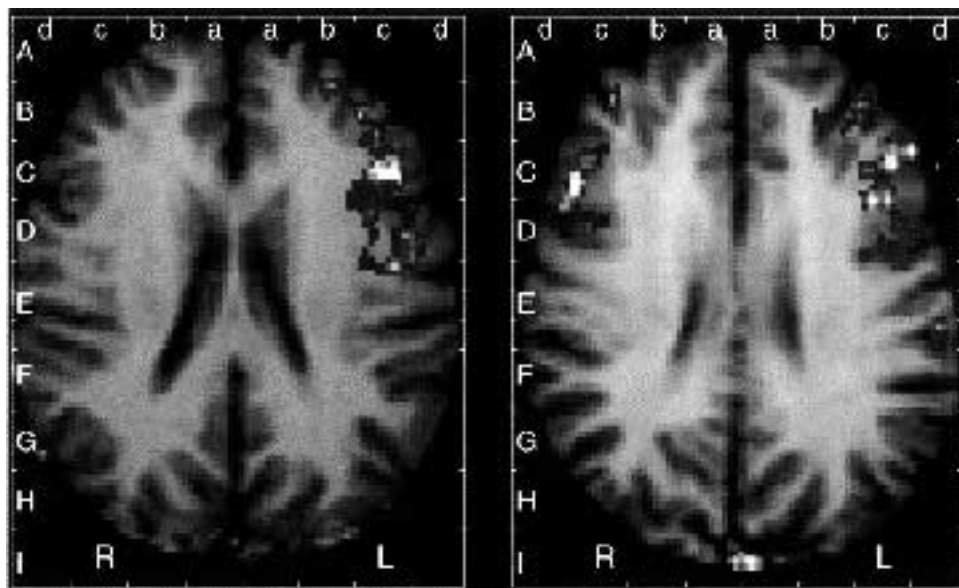
A much debated question is whether sex differences exist in the functional organization of the brain for language (1–4). A long-held hypothesis posits that language functions are more likely to be highly lateralized in males and to be represented in both cerebral hemispheres in females (5, 6), but attempts to demonstrate this have been inconclusive (7–17). Here we use echoplanar functional magnetic resonance imaging (18–21) to study 38 right-handed subjects (19 males and 19 females) during orthographic (letter recognition), phonological (rhyme) and semantic (semantic category) tasks. During phonological tasks, brain activation in males is lateralized to the left inferior frontal gyrus regions; in females the pattern of activation is very different, engaging more diffuse neural systems that involve both the left and right inferior frontal gyrus. Our data provide clear evidence for a sex difference in the functional organization of the brain for language and indicate that these variations exist at the level of phonological processing.

We studied neurologically normal right-handed males (mean age 28.5 years) and females (mean age 24.0 years). Subjects performed four distinct same-different tasks on visually displayed stimuli: line judgement, letter case, rhyme and semantic category. The decision (same versus different) and response components (pressing a response bulb for same pairs) of these tasks are comparable, but there is a difference in the type of linguistic information engaged by each. In the line-judgement task, subjects viewed two sets of four lines with right or left orientations, one above the other, and determined whether the upper and lower displays had the same pattern of left/right alternation (engaging visual information processing). In the letter-case judgement task, two sets of consonant strings were displayed, and subjects determined whether they contained the same pattern of case alternation (engaging both visual and orthographic processing). In the rhyme-judgement task, subjects determined whether two nonsense word strings rhymed (engaging visual, orthographic and phonological processing: subjects must map the letter strings onto phonological representations). Finally, in the semantic category task, subjects

determined whether two words came from the same semantic category (engaging visual, orthographic, phonological and semantic information). By subtracting the line from the case task, activation in regions of interest associated with orthography can be isolated; by subtracting the case from the rhyme task, phonological regions of interest can be isolated; and by subtracting the nonsense word rhyme from the semantic category task, regions of interest associated with lexical semantic processing can be isolated.

Selection of candidate regions of interest was motivated by previous neuropsychological and neuroimaging investigations of language function. Behavioural research on word recognition isolates two types of coding relevant to lexical identification: orthographic (pertaining to letter encoding) and phonological (pertaining to phoneme encoding) (22, 23). Preliminary analysis identified one region uniquely associated with orthographic processing (extrastriate, ES). A second region, located within the superior aspect of the inferior frontal gyrus, roughly encompassing Brodmann's areas 44/45 (which we term IFG) and previously shown to be activated in speech tasks when phonetic decisions are required (24, 25), was found to be uniquely associated with phonological processing on rhyme judgements. The rhyme-judgement task was also associated with activation at sites in both the superior temporal gyrus and middle temporal gyrus, areas that fall within traditional language regions. But the semantic task activated both of these areas significantly more strongly than the rhyme task, suggesting that these regions subserved both phonological and lexical semantic processing. The IFG, by contrast, was uniquely associated with phonological processing, and here we focus on the contrast between IFG and ES regions in examining sex differences.

A  $2 \times 2 \times 3 \times 3$  analysis of variance (ANOVA) was performed with the following factors: region of interest (IFG versus ES), hemisphere (left versus right), task (case versus rhyme versus semantic), and sex (male versus female). For each subject, the number of pixels showing significant changes in magnetic resonance signal intensity was computed in the initial split t-test



**Figure 65.1**

Composite images of the distribution of activations comparing rhyme-case tasks (phonological processing) for 19 males (*left image*) compared to 19 females (*right image*). Colour dots represent pixels for which the mean value of the split *t*-statistic from averaging the 19 subjects was higher than 0.4 (dark red dots are close to 0.4; yellow approaches 1.0). The images were cluster-filtered so that isolated activated pixels without at least four activated neighbours were dropped. Images were coregistered using a piece-wise warping algorithm. Six image subregions were identified as described in figure 65.2 legend and each was linearly scaled so that the anatomic reference points (the anterior and posterior commissures and midline) and brain edges aligned. Coordinates (29) were then assigned to each region. Activations are shown for level 6–7 ( $z = 20$ ) of the Talairach system (29). The Talairach reference grid has been superimposed on each image. Capital letters A–I (*y*-axis) and lower-case *a–d* (*x*-axis) designate the Talairach proportional grid system. *R* and *L* are right and left sides of brain, respectively. Sections are oriented with anterior portions at top of figure. Males show unilateral activation, primarily in the left inferior frontal gyrus (centred on coordinates  $x = 5.0$ ,  $y = 1.8$ ,  $z = 20$ ), with minor activation of the left middle frontal gyrus. In females, phonological processing activates both the left (L) and right (R) inferior frontal gyri. There is smaller activation of the left and right middle frontal gyri (centred on coordinates  $x = 3.4$ ,  $y = 4.5$ ,  $z = 20$ ) and of the left post-central gyrus (centred on coordinate  $x = 6.0$ ,  $y = -2.1$ ,  $z = 20$ ).

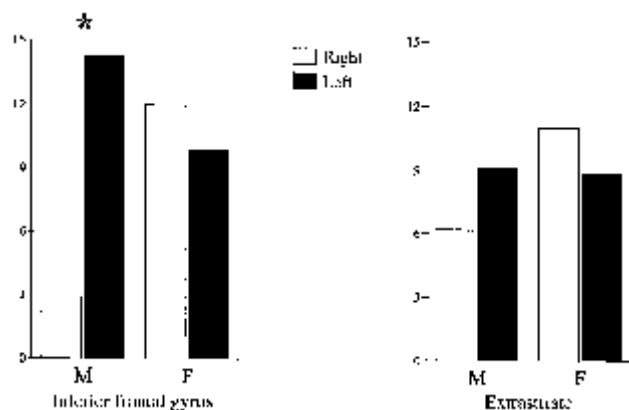
**Methods** Imaging was performed on a 1.5 Tesla GE “Signa” MR imaging system equipped with echo-planar imaging (EPI) hardware from Advanced NMR (Wilmington). Conventional spin echo sagittal  $T_1$ -weighted (TE (echo time), 11 ms; TR (repetition time), 500 ms; FOV (field of view), 24 cm; slice thickness, 5 mm; slice gap, 2.5 mm;  $256 \times 128 \times 1$  Nex (number of excitations)) localizer scans were first obtained from which axial-oblique activation images were prescribed. Three axial-oblique slices, 8 mm thick, were obtained parallel to a line connecting the anterior and posterior commissures. The inferior slice was centred at Talairach 9, the middle slice at Talairach 7–8, and the superior slice at Talairach 6–7. Conventional spin echo images (TE, 11 ms; TR, 500 ms; FOV,  $40 \times 40$  cm;  $256 \times 192 \times 2$  Nex) of these slice locations were collected before the start of each activation paradigm. These anatomic images, which are in exact registration with the activation images, were later used as the basis images on which to overlay activation maps. Subjects’ heads were immobilized within the head coil by using a neck support, foam wedges and a restraining band drawn tightly around the forehead. The calculated *t*-maps showed no significant rim artefacts or apparent activation at strong edges, confirming that head movements were not significant.

(figure 65.2) and these values were subsequently entered as the dependent measure in the ANOVA.

A significant sex-by-hemisphere interaction was observed:  $F(1, 36) = 14.74$ ,  $P < 0.001$ . For males, the mean number of pixels activated were 11.7 and 5.0 for the left and right hemispheres, respectively; the corresponding values for females were 9.4 and 12. As shown in figure 65.1, activation during rhyming in males was lateralized to the left inferior frontal regions. In contrast, activation during this same task in females engaged this region bilaterally. Error rates on each task were extremely low (on average one error per 20 trials) and did not vary systematically with task or by sex, suggesting that the tasks did not differ significantly in their difficulty. The three-way interaction between region of interest, hemisphere and sex was significant ( $F(1, 36) = 7.77$ ,  $P < 0.01$ ) and is shown in figure 65.2.

Activation in the IFG region was left-lateralized for males but bilateral for females, whereas extrastriate activation was bilateral for both males and females. In addition, these analyses confirmed that the case-line subtraction (which isolates orthographic processing) more strongly activates extrastriate sites whereas the rhyme-case subtraction (which isolates phonological processing) more strongly activates the IFG region (figure 65.3).

The regions of interest examined encompass those areas traditionally considered to be critical for language (26, 28). We recognize, however, that our study does not provide information about every possible brain region and that there may be other sites relevant to phonological processing which may not show gender differences. Although we do not want to claim that phonological processing makes no demand on right

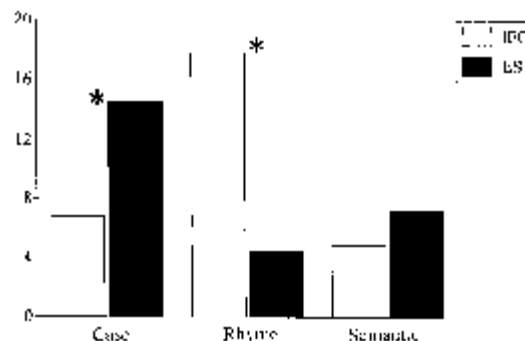


**Figure 65.2**

Three-way interaction between region of interest, hemisphere and sex. Ordinate represents mean activations across tasks for inferior frontal gyrus and extrastriate regions, respectively. Overall  $F(1, 36) = 7.77$ ,  $P < 0.01$ . For females (F), the means for left (black bars) and right (grey bars) extrastriate were 8.9 and 11.0, and for left and right inferior frontal gyrus region were 12.0 and 10.0; these means were not significantly different. For males (M), the corresponding means were 9.1 and 6.2 for left and right extrastriate, and 14.3 and 2.9 for left and right inferior frontal gyrus region. The difference for males in the inferior frontal gyrus region is significant,  $F(1, 18) = 22.34$ ,  $P < 0.001$  (indicated by asterisk). To examine this three-way interaction further, the sex by hemisphere interaction was analysed for the two regions separately. The sex by hemisphere interaction was highly reliable in the inferior frontal gyrus region,  $F(1, 36) = 20.90$ ,  $P < 0.001$  but nonsignificant in extrastriate regions ( $P > 0.05$ ). We further examined the ratio of right hemisphere to left hemisphere activation in the IFG. Eleven of 19 females but no males had a right to left hemisphere ratio  $\geq 0.70$ ; in fact for 9 of these 11 females the ratio was  $\geq 1.0$ . Thus, more than half of the female subjects produced strong bilateral activation in this region; by contrast, no males showed this pattern.

**Methods** Data analysis was performed using software written in MATLAB (Mathworks, Natick, MA). The activation images were collected using an EPI gradient echo sequence (flip angle,  $60^\circ$ ; TE, 45 ms; TR, 1,500 ms; FOV,  $40 \times 20$  cm:  $128 \times 128 \times 1$  Nex) in the three slice locations described. Twenty-four images per slice location were collected while the subject performed one of the four (line, case, rhyme or semantic) activation tasks. Each task was run 4 times, with the order of successive tasks randomized, a total of 96 images per slice per task being collected. The first seven images from each series were dropped because they were obtained before a steady state of the echo-planar sequence was reached. The remaining seventeen images from each series were median-filtered. Before median filtering, the temporal mean intensity image was subtracted from each acquisition and added back after filtering. Subject head movements were analysed but not corrected. When movements larger than one pixel were found, those image data were discarded and only the unshifted data were analysed. There was no significant artefact from motion effects at the edges that could produce false activation in functional MRI. The activated pixels were detected for each pair of activation tasks using a split Student  $t$ -test. The split  $t$ -test divides the data into two parts and performs a separate  $t$ -test on each half dataset. If the  $t$ -value for a given pixel from both  $t$ -maps was above 2, the pixel was considered to be activated. This analysis does not correct for any residual temporal correlation between successive images that can arise when the activation response varies during the task (30), but these corrections to our  $t$ -values are negligible for the steady-state response achieved during our experiments. For normally distributed data,  $t > 2$  corresponds to  $P < 0.05$ . This threshold for activation provides a consistent criterion for identifying true activity from other sources of signal variation. On each anatomical image, the positions of the anterior commissure and posterior commissure and the direction of the midline were found manually. These reference points and the edges of the brain let us define the standard Talairach coordinate sys-

tem for each subject. Each brain (anatomical image and activation map) was then rescaled to the standard Talairach form using cubic proportional fitting for each block defined by the anatomical landmarks. This procedure was remarkably successful; the major sulci and gyri can be clearly recognized on the composite image obtained by adding 38 Talairach-scaled anatomical images. Finally, each anatomical region of interest was identified in the Talairach coordinate system and approximated by a set of squares (figure 65.1). The number of activated pixels in each region was then used as a measure of the level of activation for any pair of tasks.



**Figure 65.3**

Task by region of interest. Ordinate represents mean activations for case, rhyme and semantic subtractions in the inferior frontal gyrus (IFG; grey bars) and extrastriate (ES; black bars) regions, respectively. A significant interaction between task and region was observed,  $F(2, 72) = 9.94$ ,  $P < 0.001$ . The means for the case, rhyme and semantic subtractions in the IFG region were 6.8, 17.8 and 4.9; corresponding means in the extrastriate region were 14.5, 4.5 and 7.3, respectively. Separate contrasts revealed that in the IFG region rhyme significantly differed from both case,  $F(1, 36) = 10.0$ ,  $P < 0.001$ , and semantic,  $F(1, 36) = 13.88$ ,  $P < 0.001$ , whereas case and semantic did not differ ( $F < 1.0$ ). In the extrastriate region case significantly differed from both rhyme  $F(1, 36) = 8.37$ ,  $P < 0.001$ , and semantic,  $F(1, 36) = 4.27$ ,  $P < 0.05$ . The rhyme and semantic conditions did not differ ( $F < 1.0$ ). To test the hypothesis further that extrastriate areas subserve orthographic processing while the IFG region subserves phonological processing, we contrasted activation produced in a rhyme-case versus a rhyme-line subtraction. By the logic of the design, the former subtraction differs only in phonology whereas the latter subtraction differs in both orthography and phonology. A significant difference between these two subtraction conditions should therefore be observed in the extrastriate as only the rhyme-line should isolate orthography and there should be no difference in the IFG region as both conditions should isolate phonology. As expected, the effect was significant in the extrastriate area,  $F(1, 36) = 17.89$ ,  $P < 0.001$ . The means were 4.5 and 19.0 for the rhyme-case and rhyme-line conditions, respectively. In the IFG region, the contrast was not significant ( $P > 0.10$ ) with means of 17.6 and 23.1 for the rhyme-case and rhyme-line conditions, respectively. Asterisks indicate tasks that significantly differ between regions ( $P < 0.001$ ).

hemisphere sites in males, we wish to emphasize that in a site uniquely serving phonological processing, the IFG, females devote greater right hemispheric resources to the task.

Our results indicate that it is now possible to isolate specific components of language and, at the same time, to relate these language processes to distinct patterns of functional organization in brain in neurologically normal individuals. Using this strategy, we have demonstrated remarkable differences in the functional organization of a specific component of language, phonological processing, between normal males and females. Future studies designed to examine either gender differences in language function or the neural mechanisms related to language, for example, should be specific for the component of language assessed and determined in both males and females.

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## Introduction

A large number of studies have reported sex differences in language processing. Effects are often small and superior performance has been noted in both sexes depending on the task (see Halpern, 1992, for a review). Lexical-decision (LD) tasks have consistently revealed sex differences. For example, Boles (1984) observed on a divided visual field LD task that there was a right visual field advantage (RVFA; i.e., faster reaction times to stimuli presented in the right visual field) in males that was absent in females. These results were accentuated when subjects were asked to respond both quickly and accurately. In a replication of this study, Cornier and Stubbart (1991) reported that a sex difference was only observed when a speed–accuracy task instruction was given and not when either speed or accuracy were emphasized independently. Again, males but not females had a RVFA. The results of this study are particularly noteworthy, since a speed and accuracy instruction is standard when carrying out lexical-decision experiments (see also Healey et al., 1985, and Eviatar et al., 1990, for further evidence of sex differences on lexical-decision tasks and Nieto et al., 1999, for a study showing sex differences in the processing of different word types).

Previous imaging studies have reported conflicting results: Some PET studies have failed to show sex differences in the functional activation pattern for language tasks (Buckner et al., 1995; Price et al., 1996). Buckner et al. (1995) employed a word stem completion task compared with visual fixation and a verb generation task compared with noun reading; such verbal production tasks had previously been reported to show behavioral differences between the sexes (Halpern, 1992). Other studies have suggested that consistent differences lie in the hemispheric lateralization of verbal abilities. For example, Shaywitz et al. (1995), using fMRI, showed that during a phonological processing task (i.e., subjects had to decide whether two nonwords rhymed), females produced symmetrical activation in the frontal lobes while males generated a left-lateralized response. There was also behavioral

evidence for phonological processing differences across the sexes. In a later elaboration of this study using the same subjects, Pugh et al. (1996) also showed that females showed symmetrical and males left-lateralized frontal lobe responses to a lexico-semantic processing task. The authors concluded that identical language processes are organized differently in the cerebral cortex across the sexes. However, Frost et al. (1999) replicated Pugh et al.'s study design using a large group of subjects and found no evidence for behavioral or imaging gender differences. Both sexes showed a strongly left-lateralized response during phonological and lexico-semantic tasks. Last, Jaeger et al. (1998), using PET, established that during a past-tense generation task men showed greater left-lateralized activation while women recruited bilateral perisylvian cortex. The data gathered so far are equivocal, with both positive and negative findings arising out of imaging studies that use a behavioral task with an established sex difference. While this might be explained by differences in methodology and task requirements, or simply the unreliability of measures, it would appear sensible to explore sex differences in functional cerebral laterality using clearly defined tasks with predictable effects.

From previous imaging results in the literature, we can conclude that it is important to investigate a linguistic task that shows a replicable sex difference. At present, divided-visual-field versions of the lexical-decision paradigm seem the most robust and comprehensively studied (see Fairweather, 1982, for a review), with Hiscock et al. (1995) concluding from an extensive analysis of auditory and visual laterality studies that there is a population-level sex difference supporting the hypothesis of greater lateralization in males. To date no functional imaging study has been reported for a divided-visual-field version of the lexical-decision task. This study aimed to examine whether there was a difference in functional activation pattern between the sexes for a lateralized version of a lexical-decision task (i.e., a variant of the standard lexical-decision task) and whether there was any correlation in the strength and distribution of neuronal activity with the simultaneous behavioral measure of reaction time. We used a

task instruction that stressed the importance of both speed and accuracy to emphasize any potential difference between the sexes.

## Methods

### Subjects

Twelve right-handed (Edinburgh Inventory; Oldfield, 1964), healthy native English-speaking, normal subjects were studied. There were six males and six females, with mean ages of 33.5 years ( $SD$  10.9) and 30.0 years ( $SD$  10.1) respectively.

### Experimental Procedures

**Lexical Visual Field Task** All subjects performed two experimental runs of the lexical visual field task; this was to improve the reliability of the measures. In both, two contrasting conditions were presented in alternating 30-s phases over 5 min. In epoch A there was the lexical visual field task, which was contrasted with epoch B, a motor control task. The stimuli for the two runs of lexical visual field task were 100 target real words + 100 pseudowords (50 targets and 50 pseudowords presented in each run). In both runs of the lexical visual field task a series of orienting XXX's was presented centrally for 200 ms initially, followed by both the target word and the pseudoword, which were randomly presented on either side of fixation for 2000 ms. The same basic procedure was repeated in both runs of the motor control task; a series of orienting XXX's was presented centrally for 200 ms initially, this time followed by the words PRESS + STOP; again, these stimuli were randomly presented on either side of fixation for 2000 ms. The word PRESS was the correct target for the motor control task. For both contrasting conditions (lexical visual field and motor control), in one run for each trial there was no gap between XXX and target presentation (i.e., 200-ms stimulus onset asynchrony, SOA) and an 800-ms gap after the target.<sup>1</sup> In the other run for each trial there was an 800-ms gap between XXX and target presentation (i.e., 1000-ms SOA) and no gap between trials. Hence the two runs both entailed 3 s/trial. By designing the experiment as described above, we believe the lexical visual field and motor control components were matched in terms of the screen layout, the amount of visual material presented to the subject, and the output of response, i.e., button press. Thus, the two contrasting conditions differed only in terms of lexico-semantic processing. The two experimental runs were counterbalanced across subjects.

The screen was approximately 2.4 m from the subjects' eyes and the stimuli were 3 cm in height. The stimuli were presented approximately 4° lateral of cen-

tral fixation. The words chosen in the experiments were carefully matched for length, frequency (between 10 and 100 words per million from Kucera & Francis, 1967), imageability, and concreteness from the MRC Oxford Psycholinguistic database. Subjects were asked to indicate on which side of the screen they saw the real target word or the word PRESS in the off condition. They did this by pressing one of two adjacent buttons on a pad held in their right hand (i.e., dominant hand). They were asked to respond as quickly and as accurately as possible. Pseudowords were pronounceable legally spelled letter strings and matched the words used on mean letter length.

### fMRI

**Image Acquisition** Gradient-echo, echo-planar MR images were acquired using a 1.5T GE Signa system with Advanced NMR operating console. For both experimental runs whole-head images depicting BOLD contrast (Kwong et al., 1992) were acquired with an in-plane resolution of 3 mm every 3 s. The imaging sequence of 14 near-axial noncontiguous 7-mm slices with 0.7-mm gaps parallel to the AC-PC (TE = 40 ms, TR = 3 s) was repeated 100 times over 5 min, yielding 100 brain images. An inversion recovery EPI dataset was also acquired. This was a 43 near-axial slice image, with 3-mm slice thickness and 0.3-mm slice skip parallel to the AC-PC (TE = 80 ms TI = 180 ms, TR = 16 s), used for subsequent registration.

**Image Analysis** Image datasets were initially corrected for head motion (Bullmore et al., 1999a). The physiological response to periodic experimental stimulation (ABAB, etc.) was estimated by sinusoidal regression analysis of each motion-corrected fMRI time series (Bullmore et al., 1996a). The regression model included a pair of sine and cosine waves at the (fundamental) frequency of alternation between A and B conditions, parameterized by coefficients  $\gamma$  and  $\delta$ , respectively. If  $\gamma > 0$ , the maximum fitted signal will occur during the first condition; if  $\gamma < 0$ , the maximum fitted signal will occur during the second condition (Bullmore et al., 1996b). The model was fitted by a generalized least-squares procedure, treating the residuals of a preliminary ordinary least-squares fit as a first-order autoregressive [AR(1)] process. The power of response was estimated at each voxel by the sum of squared coefficients  $\gamma^2 + \delta^2$ ; this sum was divided by its standard error to give the standardized power or fundamental power quotient (FPQ). Maps representing standardized power of response at each voxel were registered in standard stereotactic space (Talairach & Tournoux, 1988) and smoothed by a 2D Gaussian filter ( $SD$  = 5 mm).

Permutation testing was used to identify voxels demonstrating significant median standardized power of response (median FPQ) over all subjects during each task with one-tailed probability of false positive error  $p = .001$ , as described in detail by Brammer et al. (1997). Briefly, each time series in the “observed” images was randomly permuted and the sinusoidal regression model described above was fitted by generalized least-squares after permutation. This procedure generates an “estimate” of standardized power under the null hypothesis that estimated FPQ is not determined by experimental design. Repeating the procedure 10 times at each voxel generates 10 maps of FPQ estimated under the null hypothesis for each subject. All of these maps were transformed into standard space and smoothed by a 2D Gaussian filter, exactly as described for the observed maps. Finally, the null distribution of median standardized power of response (median FPQ) was sampled by computing the median FPQ, over all subjects, at each voxel for each set of permuted FPQ maps. The median FPQ observed at each voxel was tested against critical values of this permutation distribution corresponding to a one-tailed probability of type 1 error,  $p = .001$  (i.e., insuring that the FPQ values are statistically different from those in a dataset not related to the stimulus presentation). Voxels that passed this test for statistical significance are referred to as generically activated voxels because they demonstrated significant standardized power of activation by the experiment over all members of the group of subjects studied. Generically activated voxels were color coded according to the timing of the maximum MR signal (normalized by subject) of periodic stimulation with respect to the experimental input function, as described above, and superimposed on an inversion recovery EPI dataset to form a generic brain activation map (GBAM) (Brammer et al., 1997).

The main rationale for this nonparametric mode of inference is that the sample size is not large and therefore the mean standardized power of response might be unduly influenced by one or two extreme values. The median is a more robust measure of central location in small samples but its null distribution is not readily tractable. Using a permutation test, as described above and by Brammer et al. (1997), readily allows us to assess significance of group or generic activation using the median standardized power of response as our test statistic. For a fuller discussion of permutation testing in human brain mapping see Bullmore et al. (2001). There are also general introductory texts on permutation testing and related methods of computational inference by Manly (1991), Good (1994), and Edgington (1995).

Differences between male and female groups in the mean standardized power of response to the lexical visual field task were estimated by one-way analysis

of variance (ANOVA). More specifically, we fitted the following ANOVA model at each intracerebral voxel of the standardized power maps in Talairach space:

$$FPQ_{i,j,k} = \beta_0 + \beta_1 \text{Sex}_{i,k} + \varepsilon_{i,j,k}.$$

Here  $FPQ_{i,j,k}$  denotes the standardized power of response at the  $i$ th voxel in the  $j$ th member of the  $k$ th gender group;  $\beta_0$  is the overall mean power at the  $i$ th voxel;  $\beta_0 + \beta_1$  is the mean power in the  $k$ th gender group (male or female) at the  $i$ th voxel; and  $\varepsilon_{i,j,k}$  is a subject-specific error. To identify voxels at which there was significant difference between gender groups in the mean power of response, the coefficient  $\beta_1$  was tested by another permutation test with voxelwise two-tailed probability of false positive,  $p = .001$ ; for full details of this procedure and its validation see Bullmore et al. (1999b). To minimize the size of the multiple comparisons problem, voxels were only tested for a group difference if median standardized power of response was significantly large, i.e., they had been identified as generically activated voxels by the generic brain activation mapping procedure (described in detail above) in one or both of the gender groups.

## Results

### Behavioral Data

Accuracy was high for both experimental runs (<3% errors), with no significant difference between each run. There was also no difference in accuracy between the male and female groups. Reaction times to lexical visual field stimuli were slower than those in the control task (i.e., responding to which side of the screen the word PRESS occurred) [in milliseconds, mean (SD)], i.e., 892(17) versus 636(17) (there was no significant difference across experimental runs). Next, we calculated the mean reaction time for words presented in the right visual field versus those in the left visual field; reaction times for the men were 917(18) and 929(18) and for the women 892(18) and 834(19) for right and left visual fields, respectively. Thus, the men showed a nonsignificant right visual field advantage and the women a significant left visual field advantage [paired  $t$  tests: men  $t(1, 5) = -0.45$ ,  $p = .67$ ; women  $t(1, 5) = 2.55$ ,  $p = .05$ ]. Analysis of variance revealed no significant main effect for visual field [ $F(1, 10) = 1.63$ ,  $p = .23$ ] or sex [ $F(1, 10) = 0.33$ ,  $p = .57$ ], but a trend toward an interaction between visual field and sex [ $F(1, 10) = 3.89$ ,  $p = .077$ ]. However, one-way analysis of variance of the laterality coefficients ( $L - R/L + R$ ) between males (mean =  $-0.00073$ ,  $SD = 0.003$ ) and females (mean =  $0.00347$ ,  $SD = 0.003$ ) revealed a significant difference between the two groups [ $F(1, 10) = 4.88$ ,  $p < .05$ ], reflecting the different field advantages across the sexes.

**Table 66.1**Areas of activation during lexical visual field performance for males and females<sup>a</sup>

Brain area	BA	R/L	x	y	z	No.
<i>Males</i>						
Fusiform gyrus	19/37	L	-35	-64	-7	41
			-38	-61	-13	37
Inferior frontal gyrus (posterior)	44	L	-40	0	26	11
Lingual gyrus	17	L	-23	-92	-7	19
Middle frontal gyrus	9	L	-40	6	37	20
		R	49	5	37	10
Primary visual cortex	19	R	32	-58	42	13
<i>Females</i>						
Fusiform/inferior-posterior temporal lobe	18/37	L	-23	-89	-13	10
		L	-38	-50	-13	14
		R	32	-44	-13	11
Inferior frontal gyrus	44/45	L	-35	0	31	9
		L	-46	25	20	10
		R	43	11	31	29
Inferior-posterior temporal lobe	37	L	-43	-61	-7	24
Middle temporal gyrus	21	R	61	-19	-2	12

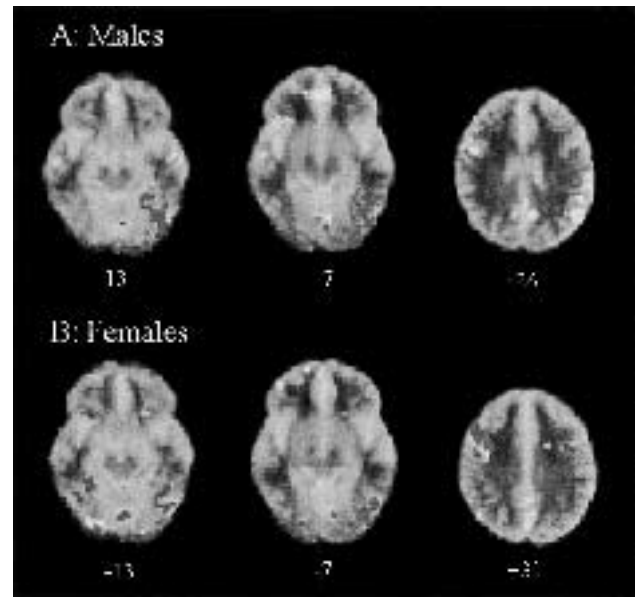
Note. BA = Brodmann Area; x, y, and z coordinates refer to the Talairach and Tournoux (1988) system; No. = number of voxels activated in the cluster.

<sup>a</sup>All activations reported in phase with lexical visual fields only (epoch A) and not the control condition (epoch B) and  $p < .0005$ .

### Imaging Data

First, we constructed generic brain activation images separately for the men and the women using data from both experimental runs. We tested whether men and women differed, taking into account the number of generically activated voxels. In total, 822 voxels were generically activated by the lexical visual field task in the male group and 591 voxels in the female group. Anatomical details of the areas of generic activation in each group are shown in table 66.1. The men showed activations in the left inferior frontal gyrus (IFG) [approximate Brodmann area (BA) 44/45], lingual gyrus (BA 18), fusiform gyrus (FG)(BA 37/18), and bilateral middle frontal gyrus (MFG)(BA 9). The female group, however, showed *bilateral* FG/inferior posterior temporal lobe (IPTL, BA 18/37) and IFG (BA 44) and right MFG (BA 9) (thus more individual regions than the male group). The male sample, therefore, showed more left-sided activation than the females and also larger regions of left-hemispheric activation; for example, in the fusiform gyrus there were clusters of 41 and 37 voxels in the male sample while only 10 and 14 voxels in the female sample (see table 66.1). The female sample also showed more right-sided activations, especially in the FG/IPTL and IFG. The main regions of activation are shown in figure 66.1.

Table 66.2 shows the results of the contrast between the two groups. The contrast revealed that the female

**Figure 66.1**

Regions of activation for the mean image of the lexical visual field experiments for (A) men and (B) women. The activation map of the mean response was derived from the 12 individual images for each sex group (i.e., 6 subjects per sex group multiplied by two runs). Activation foci are superimposed on a gray-scale template of subjects' normalized averaged high-resolution EPI images. The main areas of activation shown are the bilateral fusiform gyrus (FG), lingual gyrus (LG), inferior frontal gyrus (IFG) (BA 44/45), and the left inferior temporal gyrus (IPTL) (BA 37). Three sections are shown; these are 7 and 13 mm below the AC-PC line and 26 and 31 mm above the AC-PC line in the male and female samples respectively. The different colors indicate the phase of the GBAM; red voxels depict activation in phase with the lexical visual field task itself while the yellow voxels depict activation in phase with the control task (for brevity none of these latter regions are labeled or discussed). The right hemisphere is shown to the left of the image.

**Table 66.2**

Areas of activation significantly different when contrasting male and female samples

	BA	R/L	x	y	z	No.
<i>Greater activation in male sample</i>						
Inferior-posterior temporal lobe	37	L	-43	-53	-7	6
Middle occipital gyrus	19	L	-46	-72	-7	5
Primary visual cortex	19	R	32	-58	42	3
<i>Greater activation in female sample</i>						
Dorsolateral prefrontal cortex	9	R	49	19	26	3
Inferior frontal gyrus	44	R	40	8	31	3
Middle temporal gyrus	21	R	64	-14	-2	3

Note. Abbreviations as table 66.1.

group showed significantly more right-sided activation of the dorsolateral prefrontal cortex, IFG, and MTG. The male sample, however, showed regions of greater activation in the left IPTL, middle occipital gyrus, and primary visual regions.

## Discussion

This study confirmed that there are sex differences in functional activation on a lexical visual field task. The males in this sample showed activation that was more lateralized to the left hemisphere, including the inferior-posterior temporal lobe, fusiform, and lingual gyrus. The women, however, showed (a) more brain regions activated during the task per se and (b) greater right-sided activation, especially of the inferior frontal gyrus and inferior posterior and middle temporal gyrus (see figure 66.1). In addition there was behavioral evidence to support these imaging findings; the male sample showed marginally faster reaction times when words were presented in the right visual field and the female sample showed a left visual field advantage.

The RT data in this study correspond to the behavioral literature showing sex differences using a lexical visual field task (Boles, 1984; Cornier & Stubbart, 1991). The borderline significance of the main ANOVA can probably be explained by the comparatively small sample size used in this study (previous behavioral studies have used at least  $n > 20$ ). However, the comparison of the laterality coefficients did reveal a significant difference across the two groups. There is also a suggestion from the data that women show an overall trend to faster reaction times, although this finding would clearly need to be replicated with a larger sample.

The imaging data support the idea that men and women carry out the same linguistic processes using differently organized neural systems (Pugh et al., 1996; Shaywitz et al., 1995). The women had a more bilateral representation of language processing than the men, and the men showed a greater response to stimuli in their left hemisphere on a lexical visual field task. Our results also emphasize the importance of using a behavioral task with a well-established sex difference. In the future, it will be interesting to test this hypothesis of sexual dimorphism in lateralized representation of brain language functions by computing laterality indices from the functional imaging data in each individual and comparing mean fMRI laterality indices between males and females.

The study additionally replicates the involvement of both posterior and anterior language regions, approximately corresponding to Wernicke's and Broca's, when performing a visual lexical-decision task in both sexes

(Price et al., 1994; Rumsey et al., 1997) as well as those involved in basic visual word recognition (BA 18/19) [see Nobre et al. (1994) and Bookheimer et al. (1995) for relevant ERP and PET studies, respectively]. Activation of the inferior frontal cortex, close to Broca's area, is suggestive of engagement of rehearsal processes and the articulatory loop when making lexical decisions (Rumsey et al., 1997).

One possible criticism of this study is that the experimental run that had 800 ms between the fixation point and the stimuli could have resulted in failure of central gaze fixation. However, comparison of the behavioral and the fMRI data across the two experimental runs shows there were no major differences.

In conclusion, both the behavioral and imaging results of this study confirmed there was a sex difference on a lateralized lexical visual field task using functional MRI. The men showed activation that was more lateralized in the left hemisphere while women showed more bilateral activation when language-related regions were examined. The study does have several limitations, such as the small sample, indicating a replication is needed with a larger sample. Furthermore, a task with a different or opposite pattern of functional asymmetry (i.e., right-hemisphere dominance) would be necessary to confirm the task-specific nature of the lexical visual field results shown here.

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## Note

1. The experiments were designed this way to parallel two semantic priming tasks carried out by the investigators in a separate study.

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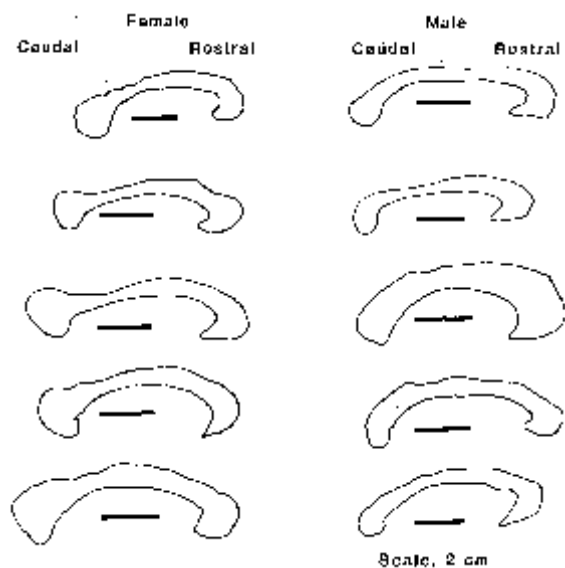
Although sex-related allometric variations in brain weight have been reported (1), to our knowledge no reliable sex differences in human brain morphology have been evidenced to date (2). In examining corpora callosa, we observed a sex difference in the shape of the splenium—the caudal or posterior portion of the corpus callosum. This serendipitous finding, later quantitatively substantiated, is of pragmatic interest to the forensic scientist. In addition, it has wide-ranging implications for students of human evolution and comparative neuroanatomy, as well as for neuropsychologists in search of an anatomical basis for possible gender differences in the degree of cerebral lateralization. To our knowledge, the existence of sexual dimorphism in the major cerebral commissure has not been reported and thus has promise for future research on anatomical sex differences in the human brain.

Whole normal brains [ $N = 14$ : male (M) = 9, female (F) = 5] were obtained upon autopsy (3). All of the brains were suspended by their basilar artery in a 10 percent Formalin-saline solution (4) for a minimum of 3 weeks and cut midsagittally, precisely through the cerebral aqueduct. Kodachrome slides were taken of the medial aspect of the brains along with a millimeter ruler. The slides were back-projected onto a glass table, and the outline of the corpus callosum was drawn at a magnification of 1.7 to 2.2. Drawings of the corpora callosa were used for (i) gross morphological examination; (ii) computation of the anterior-to-posterior distance (callosal length) and maximum splenial width (5); and (iii) computer-assisted planimetric measurements of the total callosal cross-sectional surface area as well as of the partial areas of the posterior fifth, fourth, and third of the corpus callosum. The partial surface areas, which were determined on the basis of the anterior-to-posterior distance, were used as an objective quantification of splenial area since there is no natural division between the body and splenium of the corpus callosum. All of the measurements were obtained without any information on the sex, age, brain weight, and so forth, of the individual (6).

Gross morphological examination (figure 67.1) revealed a sexual dimorphism in the shape of the sple-

nium. The female splenium is bulbous and widens markedly with respect to the body of the callosum. In contrast, the male counterpart is approximately cylindrical and is relatively continuous in width with the body of the corpus callosum. All drawings were correctly classified according to sex by three impartial observers on the basis of a verbal description of the sex differences.

A quantitative analysis of the maximum splenial width yielded a nearly bimodal distribution for males and females and confirmed the visual observations ( $t = -5.03$ ;  $P < .001$ ) (table 67.1). No sex differences were found in the absolute length of the corpus callosum. However, planimetric measurements did evince a sexual dimorphism: The average area of the posterior fifth (determined to be most representative of splenial surface area) of the corpus callosum was larger in females than in males ( $t = -1.85$ ;  $P = .08$ ), and although the average total callosal area was not



**Figure 67.1**

Sexual dimorphism in the splenium. The female splenium is more bulbous and larger than the male counterpart. All female callosa ( $N = 5$ ) used in this study are displayed; the males were randomly selected from the total sample ( $N = 9$ ).

**Table 67.1**

Quantitative aspects of sexual dimorphism in the human callosum

Sex Descriptors	Maximum Splenial Width (cm)*	Area (mm <sup>2</sup> )		Brain Weight (g) <sup>‡</sup>
		Posterior Fifth <sup>†</sup>	Total	
<i>Male</i>				
Mean	1.14	186.1	704.3	1379.4
Standard deviation	0.174	24.9	131.5	90.7
Standard error	0.058	8.3	43.8	30.2
Range	0.9 to 1.41	155.9 to 243.1	578.6 to 962.1	1220 to 1520
<i>Female</i>				
Mean	1.64	218.3	708.3	1205.0
Standard deviation	0.182	41.1	116.6	170.7
Standard error	0.081	18.4	52.2	76.4
Range	1.4 to 1.8	148.9 to 258.6	533.4 to 845.0	1090 to 1500

Statistical comparisons between male and female measurements were made with two-tailed *t*-tests. \**t*(12) = -5.03, *P* < .001. †*t*(12) = -1.85, *P* = .08. ‡*t*(12) = 2.54, *P* = .026.

absolutely larger in either sex, it was greater in females relative to brain weight (table 67.1). In addition, the total surface area of the callosum correlated significantly with splenial width in females ( $r_s = .74$ ; *P* = .03) but not in males ( $r_s = .35$ , *P* = .10). A discriminant analysis (7) using total callosal area, maximum splenial width, and surface areas of the posterior fifth and third as variables classified the callosa as male or female with 100 percent accuracy. Partial correlations of maximum splenial width with sex by brain weight, body weight, height, and age accounted for very little of the variance (*P* > .1). Therefore, the relationship between maximum splenial width and sex cannot be explained on the basis of these variables.

We can only speculate on the functional significance of the sex differences. We know from topographic studies in rhesus monkeys (8) as well as in the human brain (9) that parietal, peristriate, and some superior temporal fibers course through the splenium of the corpus callosum. Animal studies in the cat (10), rhesus monkey (11), and chimpanzee (12), as well as data from human partial commissurotomies (13), have evidenced the role of the splenium in the interhemispheric transfer of visual information. If we are to believe that a larger splenium implies a larger number of fibers interconnecting cortical areas and that the number of interhemispheric fibers correlates inversely with lateralization of function, then our results are congruent with a recent neuropsychological hypothesis that the female brain is less well lateralized—that is, manifests less hemispheric specialization—than the male brain for visuospatial functions (14). Our results need to be replicated with a larger sample size, and we await quantitative ultrastructural information on the relative numbers and density of myelinated and unmyelinated fibers in the splenium as well as more refined neuropsychological tests before we can interpret further this correlation

of our anatomical finding with neuropsychological observations.

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2. Sex differences in brain morphology have been reported in nonhuman animals [for example, F. Nottebohm and A. P. Arnold, *Science* 194, 211 (1976); R. A. Gorski, J. H. Gordon, J. E. Shryne, A. M. Southam, *Brain Res.* 148, 333 (1978)].
3. Autopsies were performed at the Dallas Forensic Institute. One of us (C.L.U.) is analyzing another sample of adult normal callosa (*N* = 14; *M* = 6; *F* = 8) obtained by J. B. Kirkpatrick at the Baylor College of Medicine, as well as a sample of fetal callosa (*N* = 38; *M* = 20; *F* = 18) from the Yakovlev collection of the Armed Forces Institute of Pathology. Preliminary planimetric measurements confirm most of the observations reported in this report.
4. Saline was mixed with the Formalin to ensure that the brains floated and retained their original shape.
5. Maximum splenial width refers to the width obtained in the widest portion of the splenium. The line drawn representing this width had to be perpendicular to parallel lines drawn along the dorsal and ventral aspects of the splenium.
6. Although the partial surface areas were computed after we had noted the sex difference, all of the drawings and calculations of the total callosal surface area were completed before we suspected the existence of such a difference.
7. The "Discriminant" subprogram of SPSS [N. H. Nie, C. Hadlai-hull, J. G. Jenkins, K. Steinbrenner, D. H. Bent (*Statistical Package for the Social Sciences* (McGraw-Hill, New York, 1975))] was used. The mathematical objective of the discriminant analysis is to weight and linearly combine a set of variables, selected by the researcher, to maximize the statistical distinctiveness of the two or more groups. In this analysis, the unstandardized canonical discriminant function coefficients (these give the weights of the discriminating variables) were total callosal area, -1.9474; maximum splenial width, 0.97378; area of the posterior fifth, 0.79470; and area of the posterior third, -0.02357.
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  15. This research was conducted at the University of Texas Health Science Center at Dallas. We thank J. B. Kirkpatrick and E. D. Ross and the Biological Humanities Foundation.
- \* Present address: Department of Cell Biology, University of Texas Health Science Center, Dallas 75235.



Neuropsychological studies suggest that males show greater cerebral lateralization of language and spatial skills, whereas females evidence greater bilateralization of function, particularly for language (1). Sex-related anatomical differences between the cerebral hemispheres have also been reported. Wada and associates (8) found that the left planum temporale—a brain region closely associated with language skills (2)—is likely to be larger in adult men than in adult women.

Recent interest has focused on morphological sex-related differences of the corpus callosum. deLacoste-Utamsing and Holloway (6) reported greater splenial width and area (the posterior fifth of the callosum), but not greater callosal length, in 5 female versus 9 male cadaver brains. In addition, the total cross-sectional area, although indistinguishable as an absolute value, was larger in the females when calculated relative to brain weight.

Witelson (9), who examined 12 male and 21 female cadaver brains, found no sex-related difference in callosal or splenial areas. Consistent with the previous finding (6), however, females had a larger callosal area only when calculated relative to brain weight. Witelson also reported that ambidextrous subjects had a slightly larger callosal area than those who were right-handed ( $0.75 \text{ cm}^2$ ), but this difference was unrelated to sex.

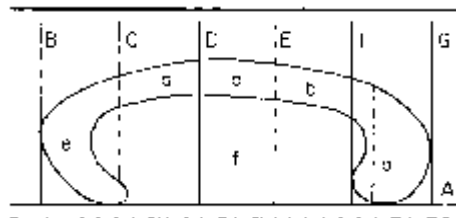
deLacoste-Utamsing and Holloway (6) defined the splenium by dividing the maximal callosal length into fifths and calculating the area of the most posterior fifth. They argued that the female splenium is more bulbous, but offer no quantitative evidence. Alternatively, a larger splenium might be observed if the callosum had a greater curvature. Since the area subtended by an arc increases with the curvature of the arc, a callosum with a greater curvature should be subtended by a larger area.

The poor replication and uncertain interpretation of these earlier studies indicated the need for a larger study. Magnetic resonance imagery (MRI) offers the advantage of an *in vivo* analysis on a larger number of subjects. In this study, MRI was used to examine callosal morphology for several sex-related differences.

## Method

The sagittal images evaluated were produced by a 0.5-T Teslacon MRI system at the Cornell University Medical Center (see [5]). The subjects, 40 men and 40 women, were patients at the New York Hospital–Cornell Medical Center, and were suffering from diseases that did not involve either the cerebral hemispheres or the corpus callosum. The MR images had a nominal in-phase resolution of  $1.0 \times 1.3 \text{ mm}$ , enabling detailed observation of callosal morphology.

Tracings of the MR images were enlarged, and morphometric area measurements were made using a Zeiss MOP-3 X;Y image digitizer. The methods of measurement previously employed (6, 9) were not accurately described; however, a similar method was estimated. Measured areas were determined as follows (figure 68.1). A straight line (line A) was drawn through the most inferior borders of the splenium and rostrum. From this line, perpendicular lines were drawn at the anterior edge of the rostrum and at the caudal end of the splenium (lines B and G). The length of line A between lines B and G represented the maximal callosal length. This length was divided by five and further perpendicular lines were then drawn (lines C, D, E, and F) to then divide the callosum into five areas (a, b, c, d, e). Measurements were also made of the total callosal area and of the area beneath the callosum but above line A (area f). In places where the splenium or rostrum curved from an external subregion into an internal subregion (e.g., from BC to EF), the brain



**Figure 68.1**  
 Method of measurement. (See text for further description.)

**Table 68.1**  
Statistical results

	Callosal Areas <sup>a</sup>						Width
	a (Splenium)	Body			e (Anterior)	f (Subcallosum)	
		b	c	d			
<i>Male</i>							
Mean (%)	30.4	11.4	12.6	13.0	31.2	150.6	23.7
SD	4.05	2.59	1.77	2.44	3.64	32.27	4.15
<i>Female</i>							
Mean (%)	31.2	11.0	12.4	13.2	30.4	148.5	24.1
SD	3.39	1.69	1.92	2.90	3.72	36.31	2.55
<i>t</i> ( <i>df</i> = 78)	-0.923	0.968	0.551	-0.436	0.985	0.270	-0.516
<i>p</i>	NS	NS	NS	NS	NS	NS	NS
Standard error	0.834	0.488	0.413	0.599	0.822	7.68	0.770

<sup>a</sup>See text and figure for description of areas.

SD = standard deviation; NS = not significant.

region involved was still considered to be part of the external subregion.

Since size reduction of the MR images and enlargement of the tracings made ascertainment of absolute callosal areas difficult, all areas were calculated as a percentage of the total callosal area for each subject. Splenium width was measured perpendicular to line A at the point in area a where the inferior to superior width was greatest (see dotted line in the figure). Again, because of enlargement differences, this width was expressed as a percentage of maximal callosal length. Since both deLacoste-Utamsing and Holloway (6) and Witelson (9) agree that there are no sex-related differences associated with either total callosal area or maximal callosal length, a sex-related difference in splenium size or width should be evident when expressed as a percentage of the total area or length.

## Results

Table 68.1 shows the mean measurements obtained from 80 subjects (40 male, 40 female) for each of the callosal fifths as well as the area beneath the callosum and the maximal splenial width. The values for callosal areas *a* through *f* are expressed as the mean of the percentage of total callosal areas. The mean widths were calculated as percentages of the maximal callosal lengths.

Although both splenial area and width tended to be larger in females, no significant sex-related differences for any of the measurements were found ( $p > 0.05$ ).

## Discussion

The results of this study reveal no sex-related difference in the percentage of callosum occupied by the splenium

or any of the other callosal fifths, splenial width, splenial shape, or callosal curvature. deLacoste-Utamsing and Holloway's report of sex-related differences in splenial parameters must be viewed in the context of their small sample size as well as in the context of Witelson's (and the present) failure to confirm these findings in larger samples.

deLacoste-Utamsing and Holloway (6) found that females had a larger callosum only when size was calculated relative to brain weight. In other words, male brains weighed more than female brains. Such sex-related differences in brain weight are well known (see [4]), and it is not clear that this should be related to callosal area. Because *in vivo* brain weight was not quantified, we could not test this finding.

Our examination of callosal curvature also failed to confirm the possibility of a sex-related difference in the area subtended by the callosum (area *f*). deLacoste-Utamsing and Holloway have claimed that impartial observers could categorize callosal outlines according to sex based on the marked bulbous morphology of the corpus callosum in females (versus the cylindrical shape in males). A similar qualitative test was conducted by a single observer who was familiarized with the previous morphological criteria. His scores were at chance level (23 of 40 were sorted incorrectly). Furthermore, he was no better at sorting either the female or male callosal outlines.

deLacoste-Utamsing and Holloway argued that a larger splenium would correlate with a larger number of interhemispheric fibers and less lateralization of function. However, callosal area is dependent not only on the total number of crossing fibers, but also on the density of these fibers. Tomasch (7) found great variance in the distribution of fiber densities across the human corpus callosum. Therefore, further cadaver

investigations of sex-related callosal differences should include measurements of axonal densities.

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Aside from absolute and relative brain weights (e.g., Holloway, 1980), sexual dimorphism in the human CNS has not been well documented except for the hypothalamus (Swaab and Fliers, 1985; Hines et al., 1992; see Breedlove, 1992, for a general review on vertebrate CNS dimorphism). Midline structure dimorphism was suggested by Papez (1937) while he was studying the brain of Helen Gardner, but the original measurements were not published, precluding statistical analysis. Bean (1906) suggested both sexual and ethnic differences in the corpus callosum as well as the rest of the brain, but a later study by Mall (1909) found little of substance, except brain size as being truly dimorphic. Many references regarding cerebral hemispheric dominance organization for language and spatial abilities strongly suggest some sexual dimorphism (e.g., Kimura, 1987, 1992; Hines et al., 1992), but the question of whether the differences are inborn or culturally acquired is controversial (e.g., Maccoby and Jacklin, 1974; Kimura and Harshman, 1984; Harris, 1978; Juraska, 1986; Toran-Allerand, 1986; in particular see McGlone, 1980, and peer commentary). Most current opinion seems to favor the genetic position while recognizing the importance of culturally mediated social behavior in the maturation of the CNS. Indeed, Juraska and Kopcik (1988) have demonstrated that handling and hormonal mediation can affect both the size of the rat corpus callosum and the distribution of its fibers (see also Berrebi et al., 1988, and Deneberg et al., 1991, below).

Elsewhere, one of us (RLH) has speculated that there is an anatomical basis for sexual dimorphism in certain aspects of cognitive functioning, and this dimorphism is evolutionarily derived based on natural selection for complementary social behaviors in adults related to nurturance of dependent offspring (Holloway, 1983, 1990).<sup>1</sup> In essence, females devote more energy to social communication involving the inferior parietal and inferior temporal lobes, and thus use both cortices to a greater degree than males, whose dominant parietal lobe appears to be more specialized for visuospatial abilities. Furthermore, at least one of us (RLH) believes that the difference may be species spe-

cific (Holloway, 1986, 1990), as thus far sexual dimorphism in the relative sizes of the corpus callosum (CC) does not appear in the Anthrozoidea, although the pongids have not been adequately studied thus far (Holloway and Heilbroner, 1992). In rats, Deneberg et al. (1991, 1992) have shown that the male rat has a larger CC than the female, and that the size differences are responsive to hormonal mediation, particularly in females.

The major structure responsible for the communication between the cerebral hemispheres is the corpus callosum (CC). Some 200 million fibers are believed to interconnect the two hemispheres through the CC (Tomasch, 1954). Recent work by Aboitiz et al. (1992) suggests no essential differences in fiber types and their distribution between human males and females.

#### Relative and Absolute Size of the Corpus Callosum

It remains our contention that there exists an important dimorphism between human males and females, such that the female corpus callosum is *relatively* larger than the male CC and that the difference is largest in the splenial or posterior portion of the CC, which mostly interconnects the inferior parietal, anterior occipital, and inferotemporal as well as primary visual cortex (de Lacoste-Utamsing and Holloway, 1982; Holloway and de Lacoste, 1986; de Lacoste et al., 1986). This is an important finding but one that is understandably controversial, as it provides the first evidence for anatomical differences in the human brain associated with the cerebral cortex, that is, above the level of the hypothalamus. The original findings by de Lacoste-Utamsing and Holloway (1982) describing a sexual dimorphism in the relative and absolute size of the CC favoring females appears to be the most controversial finding, particularly regarding absolute differences. While those results were basically confirmed and extended by Holloway and de Lacoste (1986, except with respect to posterior splenial one-fifth portion) and Holloway (1990), many reports claim not to have duplicated these findings (e.g., Clarke et al., 1989; Openheim et al., 1987; Witelson, 1985; Kertesz et al.,

1987; Demeter et al., 1988; Byne et al., 1988; Weis et al., 1988; Weber and Weis 1986; Habib et al., 1991; Deneberg et al., 1992).

On the other hand, Witelson (1989) has claimed some dimorphism as well as handedness effects for the so-called isthmus of the CC, which is where the posterior part of the body of the CC grades into the anterior splenial portion of the CC. The dimension appears to be larger in females, but it is not clear whether the difference is statistically significant. Elster et al. (1990) found some support for a limited but persistent dimorphism. A recent paper by Steinmetz et al. (1992) found a significant sex effect in the so-called isthmus but not one for handedness in their MRI study of 52 young adults. This finding is in contradiction to both Witelson (1985) and Deneberg et al., (1991) regarding handedness, a finding also supported by Nasrallah et al. (1986). More recently, Hines et al. (1992) have found some limited support for splenial dimorphism by studying the relationship between language and visuo-spatial cognition and the size of the splenium in a large female sample. However, males were not studied nor were their CCs measured.

As indicated in an earlier review (Holloway, 1990), many of these seemingly contradictory results were based on MRI studies where no control of brain size was provided, despite our initial insistence that it was the *relative* size of the CC which was dimorphic (deLacoste-Utamsing and Holloway, 1982). Indeed, as will become apparent (see also Appendix I), many of the statistical procedures published elsewhere have indicated near equality of CC size patterns between males and females in absolute terms. The issue of *relative* size of the CC, that is, relative to total brain size, is difficult to study as brain size is a measure with a high degree of sexual dimorphism in humans.

Only Peters (1988) tackled the problem of a lack of allometry in the size of the corpus callosum, realizing that the *relative* measures are larger for females. Both Deneberg et al. (1991) and Demeter et al. (1988) have claimed that since there is no correlation between the size of the CC and brain weight, brain size can thus be ignored. We believe this is highly unlikely, and this study shows a low but significant correlation between brain weight and most measures of the CC, except for dorsoventral splenial width, which appears to be highly dimorphic.

Papers supporting our earlier findings are rare and only partial support is given. Clarke et al. (1989) have found some limited support of our original observations regarding splenial bulbousness, but by and large their results demonstrate no significant differences in size of either the CC or its components between the sexes. While Allen et al. (1991) have also replicated

some of our findings on the shape of the CC, that is, in the splenial region, they basically find little dimorphism in total measures. In addition, work by Hines et al. (1992) offers some support for a relationship between the splenium, handedness, and cognition, with different scores in these later measures between females with large splenial regions. The Allen et al. (1991) paper is of particular interest as they used much of the same methodology as found in our original paper (de Lacoste-Utamsing and Holloway 1982), and with larger sample sizes. Their discussion of the problems is very useful. The findings of Steinmetz et al. (1992) are clearly consistent with our previous findings, but significance levels are not provided.

We do not know the reasons for each of the inconsistencies in these various reports. Several (see below and Appendix I) can be explained partially as a failure of other workers to study the *relative* size patterns, relying on a lack of statistical significance in *absolute* values to claim disproval of the dimorphism hypothesis. As Allen et al. (1991) have suggested, it is important to use similar methods when attempting to replicate results, and indeed we do believe methodology as well as goals may help to explain some of the differences in reports (i.e., cadaver vs. living MRI patients, absolute vs. *relative* measures of the CC, and the variable of handedness and its interaction effects with sex).

In addition, sampling is likely to be an important factor in generating different results. The original studies by Holloway and de Lacoste relied on autopsied brains (not MRI) and were quite small in sample size. We believed then, and do now, that such samples were too small to show the high degree of overlapping that occurs when large samples of each sex are compared, and in that sense our latest work is not totally consistent with the earliest studies by de LaCoste-Utamsing and Holloway (1982) and Holloway and de LaCoste (1986). In our experience, getting larger sample sizes has been difficult, given the preference for brain autopsy procedures to use coronal sectioning, and the current ease of acquiring MRI samples. We still regard autopsy studies as essential, however, because MRI analyses fail to provide a reliable control of brain size.

Three independent samples collected from 1985 through 1987 are reported here for the first time. The results of these three independent samples replicate our major findings. These results also show that there is considerable overlap in the size of these structures, both absolutely and relatively, and that the strength of the dimorphism may vary in different samples and populations. In addition, we will show that the patterns of sexual dimorphism in the brain for other neurologi-

cal structures are very different from what appears to be the case for the CC. We believe that such a demonstration strengthens our arguments regarding the dimorphism of the CC, and lays the burden of explanation upon other workers to explain why only the corpus callosum follows such a pattern among major neurological structures.

In this paper, we are not primarily concerned with the functional sequelae of such dimorphic differences as may exist, or their ontogenetic, endocrinological, or behavioral relationships (including handedness). Many of the references cited in this report have excellent discussions of such relationships. Given the controversies surrounding relative and absolute measures as well as handedness, and the limited sample sizes of autopsied materials, we wish to confine our study to whether or not sexual dimorphism is present in our autopsy samples, and to explain as much of the controversial inconsistency as possible. (Comparative studies may be found in Holloway and Heilbroner, 1992; Heilbroner and Holloway, 1989; de Lacoste and Woodward, 1988. More speculative appraisals may be found in Holloway, 1983, 1990.)

### Material and Methods

Three brain autopsy samples from adults were collected: (1) 47 brains (21 males, 26 females) were collected at Mt. Sinai Hospital, New York; (2) 43 brains (20 male, 23 female) were collected from Columbia University's College of Physicians and Surgeons, New York; (3) 29 brains (15 male, 14 female) were collected from Australian Aborigines in Perth, Australia. Both New York (NY) samples were multiethnic, but the large majority were Caucasian. Standard autopsy procedures were used in each institution with but minor variations. All brains were sectioned at the level of the medullocervical junction prior to weighing. Brains from Mt. Sinai were fixed by suspension in 10 percent saline/formalin, phosphate buffered to a pH of 7.2. At Columbia, brains were fixed in nonbuffered 10% formalin solution. Clinical records were examined by the pathologist at the time of autopsy to exclude cases with neurological and/or severe psychological disorders from the study, including alcohol and/or drug-related cases.

In the case of the Australian Aboriginal sample, some of the cases were accompanied by fresh brain weights and by the weight of the cerebral cortex in others. We use cerebral cortex weight in this sample for our brain size correction, as it provides the larger of the possible samples. We note that Witelson (1989) has also followed this procedure.

Brain dissections were performed after 14–28 days of fixation. Brains were weighed before dissection on a

spring-loaded scale with an accuracy of  $\pm 10$  grams. A midsagittal section was then made, passing through the plane of the midline of the CC, third ventricle, cerebral aqueduct, interpeduncular fossa, mammillary bodies, and median raphe of the brainstem.

In the first two samples, color slides were taken of the midsagittal plane, with a metric ruler in the same plane, as described in de Lacoste-Utamsing and Holloway (1982). The Australian samples was also photographed, but on black-and-white film, with a rule in the same plane. The outline of the CC was then traced onto the photograph and measured directly. The NY slides were projected through an enlarger and thus measured at higher magnifications than the Australian sample. All measurements were taken by only one of us (RLH). In no instance was the sex, age, or brain weight of the brain known until after all measurements had been compiled. These data were provided separately from the slides/photographs after measuring was completed.

The slides and photographs were processed as described in detail elsewhere (Holloway and de Lacoste, 1986; deLacoste-Utamsing and Holloway, 1982), and the CC was measured for (1) total surface area in the midsagittal plane (CCAREA); (2) the dorsoventral width of the splenial portion of the CC (SPLNDV), which is a measurement taken at a right angle to the anterior-posterior axis of the body of the splenium at its widest point; the posterior one fifth of the area of the CC, which is almost always exclusively the splenium (POST5). The maximum width of the body at the mid anterior-posterior distance between the genu and tail of the splenium was also measured.

**Table 69.1**

Absolute and relative measurements used in this study\*

Measurement	Description
<i>Absolute</i>	
CCAREA	Total cross-sectional area (cm <sup>2</sup> ) of the corpus callosum in the midsagittal plane
SPLNDV	Maximum dorsoventral width (cm) of the splenium taken at a right angle to the longitudinal axis of the splenium
POST5	Area (cm <sup>2</sup> ) of the CC included in the posterior 1/5 segment of the A-P length of the CC
<i>Relative, i.e., brain-size corrected</i>	
RELCC	CCAREA/(brain weight <sup>2/3</sup> )
POST5/CC	POST5 $\times$ 100/CCAREA
RELSPLN	SPLNDV $\times$ 100/(brain weight <sup>1/3</sup> )
RATIO1	(POST5/CC) $\times$ 100/(brain weight <sup>2/3</sup> )

\*"Relative" in this context refers to ratio data where the absolute variates such as CCAREA are divided by the size of the brain or some derivative of that size, such as the one-third or two-thirds power of brain weight. Dividing by raw brain size does not change the *t*- or *P* values in any significant sense. In the case of table 69.4, the Australian Aboriginal sample, the variates are divided by cerebral weight (one-third and two-thirds power) and not total brain weight.

**Table 69.2**  
Sexual dimorphism in corpus callosum from Columbia sample\*

Variable <sup>2</sup>	Sex	N	Mean	S.D.	Range	Student- <i>t</i> <sup>1</sup>	<i>P</i> (2-Tailed)
CCAREA	M	20	6.11	1.36	3.46–9.27	0.17	.862
	F	23	6.05	1.08	3.87–8.21		
POST5	M	20	1.50	.30	0.91–2.12	–1.08	.288
	F	23	1.60	.32	1.16–2.14		
SPLNOV	M	20	1.07	.15	0.78–0.92	–1.44	.156
	F	23	1.14	.15	1.36–1.42		
Total brain	M	20	1357	166.4	1060–1560	2.55 <sup>†</sup>	.014
	F	23	1245	119.7	1040–1520		
RELCC	M	20	5.00	.94	3.34–6.81	–0.90	.370
	F	23	5.25	.84	3.35–6.83		
RELSPLN	M	20	9.77	1.44	6.75–13.22	–2.05 <sup>†</sup>	.047
	F	23	10.64	1.32	6.58–12.97		
RATIO1	M	20	0.206	.039	0.14–0.28	–2.64 <sup>†</sup>	.012
	F	23	0.230	.019	0.20–0.27		
POST5/CC	M	20	24.75	3.10	18.62–29.94	–2.07*	.045
	F	23	26.44	3.21	22.09–31.76		
Age (yrs)	M	20	58.3	18.1	20–83	–0.81	.42
	F	23	62.7	17.6	20–88		

<sup>1</sup> Negative values of *t* distinguish variates which appear to be larger in the female sample; dagger indicate *P* < .05.

<sup>2</sup> Areas in cm<sup>2</sup>, weights in grams.

\* In this and the succeeding two tables, the variables are those described in Table 69.1. Bold and underlined values are significant at the < .05 level. The ratio or relative data start with the RELCC variate.

The following ratios were used to account for relative size differences: (1) RELCC is simply the area of the CC divided by the two-thirds power of brain weight  $\times 100$ . The two-thirds power is used to approximate an areal dimension of brain volume. Ratio data using raw brain weight gave almost identical results. (2) POST5/CC area is the posterior one-fifth of the CC (splenium) area divided by the total area of the CC  $\times 100$ ; (3) RELSPLN is the maximum dorsoventral splenial width divided by the one-third power of brain size  $\times 100$ . The power of one-third is used to approximate a linear derivative or single dimension of brain volume. (Dividing by raw brain or cerebrum weight does not alter the statistics resulting from a power relationship). (4) RATIO1 is POST5/CC divided by the two-thirds power of brain weight  $\times 100$ . Table 69.1 summarizes these measurements.

Each sample was analyzed statistically using univariate methods, and tested for sex and age effects by ANOVA. In the ANOVA procedure Type III least squares was used, with sex as the main factor and brain weight and age as covariates. In addition, to examine sexual dimorphism in other neural structures, three independent databases from the literature were used: (1) Klekamp et al. (1989) kindly provided their data of our analyses; (2) Wessely (1970); (3) Zilles (1972). These latter two databases were collected well before the controversy regarding sexual dimorphism of the CC and were examined to gain an appreciation of the variability in other human neural structures.

## Results

Tables 69.2–69.4 provide the mean values of the various measures, both *absolute* and *relative*, for each of the three samples, and the results of Student's *t*-test for significant differences of the mean. None of the samples show significant sex differences with regard to the absolute values of CC variates, except in the Australian sample. In all samples, the brain weight and/or cerebrum weight for males is significantly larger than for females, while the values of CCAREA, POST5, and SPLNDV are essentially equal for males and females, or slightly larger for females. Both SPLNDV and POST5 area from the Columbia sample are absolutely larger in females. All three callosal variates in the Australian sample are larger for females, and nearly significantly so, with SPLNDV being truly larger in a statistically significant way. In all three samples, the range of values is extensive, as can be seen in tables 69.2–69.4.

In contrast, the brain-corrected values are significantly larger in females from each of the three, although in the Columbia sample the RELCC area is larger for females, but not significantly so. POST5/CC and RATIO1 are significantly higher in the females from the NY samples, indicating that female brains show a CC which has a higher percentage of its area that is splenial. The relative values are mixed for females in the Australian sample, but do not attain statistical significance. The cerebral cortical weights

**Table 69.3**

Sexual dimorphism in corpus callosum from Mt. Sinai sample\*

Variable	Sex	N	Mean	S.D.	Range	Student- <i>t</i> <sup>†</sup>	<i>P</i> (2-Tailed)
CCAREA	M	21	6.64	.93	5.52–8.68	0.53	.599
	F	26	6.51	.73	5.84–9.14		
POST5	M	21	1.76	.24	1.43–2.51	0.07	.945
	F	26	1.75	.18	1.46–2.30		
SPLNDV	M	21	1.21	.13	0.94–1.44	–1.26	.215
	F	26	1.26	.16	0.95–1.59		
Total brain	M	21	1373	137	1110–1595	5.79 <sup>†</sup>	.000
	F	26	1175	97	970–1335		
RELCC	M	21	5.41	.67	4.33–6.60	–2.39 <sup>†</sup>	.021
	F	26	5.89	.70	4.94–7.89		
RELSPLN	M	21	10.93	1.17	8.35–12.60	–2.70 <sup>†</sup>	.009
	F	26	12.02	1.51	8.81–14.77		
RATIO1	M	21	0.195	.026	0.174–0.271	–4.43 <sup>†</sup>	.000
	F	26	0.232	.030	0.178–0.292		
POST5/CC	M	21	25.014	3.16	18.62–31.00	–2.19 <sup>†</sup>	.034
	F	26	26.800	2.43	22.89–31.76		
Age (yrs)	M	21	67.2	11.6	35–89	0.16	.87
	F	26	66.6	14.5	30–91		

<sup>†</sup>*P* < .05.**Table 69.4**

Sexual dimorphism in corpus callosum from Australian Aboriginal sample (cerebrum corrected)

Variable	Sex	N	Mean	S.D.	Range	Student- <i>t</i>	<i>P</i> (2-Tailed)
CCAREA	M	9	5.45	0.68	4.66–6.81	–2.06	.055
	F	10	6.35	0.12	4.12–8.15		
POST5	M	9	1.38	.10	1.24–1.54	–1.77	.095
	F	10	1.54	.27	1.07–1.87		
SPLNDV	M	9	1.03	.06	0.92–1.11	–2.13 <sup>†</sup>	.047
	F	10	1.17	.19	0.92–1.55		
Total brain	M	9	1021	102.7	882–1160	2.20 <sup>†</sup>	.042
	F	10	939	57.7	833–1064		
RELCC	M	9	5.44	.82	4.43–7.11	–2.64 <sup>†</sup>	.017
	F	10	6.65	1.14	4.28–8.69		
RELSPLN	M	9	10.23	0.62	9.13–11.07	–2.57 <sup>†</sup>	.020
	F	10	11.97	1.94	9.32–15.94		
RATIO1	M	9	0.53	.073	0.41–0.65	–0.72	.450
	F	10	0.56	.086	0.43–0.69		
POST5/CC	M	9	25.45	2.55	21.63–29.26	0.84	.409
	F	10	24.47	2.45	20.76–28.90		
Age (yrs)	M	9	47.9	18.2	14–79	1.608	.126
	F	10	35.4	15.6	18–63		

<sup>†</sup>*P* < .05.

**Table 69.5**  
Pearson correlations for CC variables, brain weight, and age\*

	Brain	CCAREA	SPLNDV	POST5	Age
Brain	1.00	.3482 (.0008) <sup>1</sup>	-.0516 (.6291)	.2127 (.0442)	-.1012 (.3428)
CCAREA		1.000	.5322 (.0000)	.7979 (.0000)	-.0866 (.4172)
SPLNDV			1.000	.8013 (.0000)	.0950 (.3731)
POST5				1.000	-.0502 (.6382)

<sup>1</sup> Significance level in parentheses. Values < 0.05 in italics.

\* *N* = 90, sexes combined from Columbia and Mt. Sinai.

In this and the succeeding two tables, the underlined values of the Pearson correlations are those significant at the <.05 level for the two NY samples combined, males only (Table 69.6) and females only (Table 69.7). Note the strong correlations between callosal variables but the weakness with total brain size. None of the age correlations are significant.

are significantly different between males and females in this latter sample. Other samples of whole brain weights from Australian aborigines do indicate that the male brain is significantly larger than in females (Harper and Mina, 1981; Klekamp et al., 1989). Age differences are not significant in any sample, although the NY cases are clearly more aged, averaging 60–65 years.

The width of the body of the CC showed no significant difference between males and females in any of the samples in either absolute or relative values (the latter constructed by dividing body width by the one-third power of brain weight) and is not included in the tables for reasons of space.

The ANOVA results using sex as a main factor, and brain weight and age as covariates, indicated that both sex and brain weight could be important in producing significant *F* ratios. For CCAREA, the main effect (sex) gave a *P* of .08, and a *P* = .0000 for brain weight. (Age was not significant in any of the runs.) For POST5, the *P* for sex was .0074 and *P* = .0000 for brain weight. For SPLNDV, the *P* for brain weight was .15, whereas the *P* for sex was .021. In the case of the body of the splenium, there was no significant sex factor, while the *P* for brain weight was .02.

Given the slightly different procedures used to enlarge the Australian images of the CC, ANOVA was also performed on just the two NY samples. In these runs, the results were similar, but for CCAREA the sex effect had a *P* = .097, while the *P* (sex) for POST5 was .016. SPLNDV showed no significance for either the sex effect or brain weight. In general, then, the ANOVA results strongly suggest that both sex and brain weight are important considerations in looking at the size of the CC and some of its components.

**Table 69.6**  
Pearson correlations for CC variables, brain weight, and age\*

	Brain	CCAREA	SPLNDV	POST5	Age
Brain	1.00	.5031 (.0005) <sup>1</sup>	.0374 (.8098)	.3238 (.0320)	.0525 (.7351)
CCAREA		1.000	.5774 (.0000)	.8133 (.0000)	-.0279 (.8574)
SPLNDV			1.000	.8103 (.0000)	.0362 (.8157)
POST5				1.000	-.0502 (.6382)

<sup>1</sup> Significance level in parentheses. Values < 0.05 in italics.

\* *N* = 44, males only, combined from Columbia and Mt. Sinai.

**Table 69.7**  
Pearson correlations for CC variables, brain weight, and age\*

	Brain	CCAREA	SPLNDV	POST5	Age
Brain	1.00	.1638 (.2767) <sup>1</sup>	.1189 (.4313)	.2928 (.0483)	-.2770 (.0624)
CCAREA		1.000	.5530 (.0001)	.8042 (.0000)	-.1581 (.2941)
SPLNDV			1.000	.8055 (.0000)	.1286 (.3943)
POST5				1.000	-.0284 (.8512)

<sup>1</sup> Significance level in parentheses. Values < 0.05 in italics.

\* *N* = 45, females only, combined from Columbia and Mt. Sinai.

In addition, table 69.5 shows the correlation coefficients between the combined NY samples, which number 90. (The Australian sample is not included as the method regarding data collection from photography was slightly different from the NY samples, as described under Materials and Methods.) Noteworthy are the low (but significant) correlations between brain size and the callosal measurements. SPLNDV is particularly weak and insignificant with regard to brain size, but strong with respect to CCAREA and POST5, other callosal measures. If sexual dimorphism were not strong between males and females, the correlations between SPLNDV and other measurements would be expected to be larger.

Tables 69.6 and 69.7 show the Pearson correlations and significance levels for each sex separately. In these cases, the correlations between SPLNDV and brain weight are somewhat higher but still low and insignificant. This also suggests that SPLNDV is a dimorphic variate. It is also noteworthy that the correlations between the variables and age are mostly negative, but not significantly so. These findings are at variance with the recent report by Witelson (1989) that males show a significantly higher loss in CC size than females. In our sample, it was the females who showed a higher negative correlation with age, although it was not significant in either sex. The correlations were essentially

**Table 69.8**

Variation within samples as shown by coefficient of variation (CV) and skewness (SKEW)

	CCAREA		POST5		SPLNDV		Brain	
	CV	SKEW	CV	SKEW	CV	SKEW	CV	SKEW
<i>Columbia</i>								
Males (20)	22.2	1.12	19.7	.51	12.6	.21	12.8	.43
Females (23)	17.1	-.31	19.4	.37	13.6	.74	9.6	.41
<i>Mt. Sinai</i>								
Males (21)	14.1	1.75	13.9	2.57	10.7	-.50	10.0	-.32
Females (26)	11.3	4.38	10.6	1.64	12.4	.24	8.2	-.39
<i>Australian</i>								
Males (9)	12.5	1.1	7.6	.51	6.2	-.46	10.0	.35
Females (10)	17.6	-.35	17.3	-.34	16.3	.88	6.1	.73

the same when run with the same data standardized to a mean of 0 and a S.D. of 1.0. We have no explanation for these discrepancies beyond sampling (see below).

Table 69.8 compares the values of the coefficient of variation (CV) and the standardized skewness (SKEW) for each of the measures in both males and females of each sample. In each sample, the CV can be high, that is, greater than 12%. In addition, the Mt. Sinai sample shows high skewness for both females and males in CC area. In general, the skewness coefficient is not high in the rest of the sample. We believe this sampling indicates samples that depart from a perfect normal (Gaussian) pattern. However, other tests such as Kruskal–Wallis, Kolmogorov–Smirnov, or Mann–Whitney unpaired tests provided basically the same results as the *t* tests reported in the tables: Raw values were not significant, except for brain weight, and ratio variables were significant.

*T* tests were run once again for each of the NY samples after arbitrarily removing both one male and female, each with the highest and lowest CCAREA values. The Mt. Sinai sample continued to show significant ratios in favor of female callosal values. In the Columbia sample similarly treated, the *P* values fell between 0.05 and 0.06 for the ratio variates.

Tables 69.9–69.11 clearly demonstrate that the sexual dimorphism for other neural structures, such as the thalamus, cerebellum, ventricles, etc., are significantly high, but that the ratios are not. In addition, table 69.11 shows that the absolute value of the CC is not significantly different between males and females, but when the CC is corrected for brain weight, the ratio is significantly greater for females.

## Discussion

Three independent brain autopsy samples from different groups have been studied for sexual dimorphism in the corpus callosum, measuring both absolute and relative size of CC variates. In summary, these studies

**Table 69.9**

Sex differences in the brain based on Wessely (1970) data\*

Structure	Male ( <i>N</i> = 18)	Female ( <i>N</i> = 13)	<i>t</i> -Value	Probability
Brain	1379	1231	3.54	.0014
Cerebellum	148	135	2.51	.0180
Rhombencephalon	31.8	27.9	3.69	.0009
Ventricles	16.1	12.2	1.74	.0930
Ratio cerebel. (%)	11.10	11.27	0.61	.54
Ratio rhomb. (%)	2.38	2.34	0.49	.63
Ratio ventric. (%)	1.21	1.03	1.03	.313

\*The brain weight is in grams, as are the other structures. The ratio data are derived by dividing the neural structure in question by the brain weight and multiplying by 100. These are reported as percentages. Unlike the corpus callosum, these show no significant sexual dimorphism. Values of *P* < .05 are highlighted to show the difference between absolute and relative values.

**Table 69.10**

Sex differences in the brain weights based on Klekamp et al. (1989) data for Australian Aborigines\*

Structure	Male ( <i>N</i> = 18)	Female ( <i>N</i> = 16)	<i>t</i> -Value	Probability
Brain	1276	1104	4.903	0.00002
Cerebellum	133.4	113.9	4.08	0.0002
Hippocampus	6.20	5.17	4.31	0.0001
Amygdaloid	2.74	2.36	2.39	0.024
Thalamus	13.01	11.89	2.20	0.037
Ratio cerebel. (%)	10.46	10.34	0.373	0.71
Ratio hipp. (%)	.0048	.0047	1.06	0.29
Ratio amygd. (%)	.00215	.00212	.25	0.804
Ratio thal. (%)	.010	.011	1.28	0.210

\*The brain weight is in grams, as are the other structures. These figures were calculated after receiving the data courtesy of Drs. J. Klekamp and A. Riedel. These figures do not include Caucasian brains. The sexual dimorphism is statistically significant for all the absolute values of the brain structures, but nonsignificant when corrected for brain size, that is, made relative.

**Table 69.11**  
Sex differences in the human brain based on Zilles (1972) data\*

Structure	Male	Female	<i>t</i> -Value	Probability
Brain wt.	1308	1178	5.02	0.0000
Cortex	584	526	5.03	0.0000
CCAREA	6.18	6.28	0.481	0.63
Ratio cortex <sup>1</sup>	44.7	44.7	0.019	0.984
Ratio CCAREA <sup>1</sup>	5.42	5.90	2.74	0.007

<sup>1</sup> Ratio cortex = cortical weight divided by brain weight. Ratio CCAREA = corpus callosal area divided by brain weight.

\* As in the other tables, brain weight and cortex are in grams, and CCAREA is in centimeters squared. The ratios are the structures in question divided by brain weight. Values of  $P < .05$  are highlighted to show the differences between absolute and relative values.

support earlier findings claiming sexual dimorphism in *relative* measures (e.g., de Lacoste-Utamsing and Holloway, 1982; Holloway and de Lacoste, 1986; Holloway, 1990). At the same time, these new studies also support other reports that there is often no statistically significant sexual dimorphism in absolute CC variates. Thus these studies do not entirely replicate our earlier results. We believe this is due to the much larger sample sizes tested in this study.

The three autopsy samples reported herein show the following: (1) There is considerable overlap in the size of the CC and its divisions among males and females; (2) the CC is larger in females than that for males when brain size is taken into consideration, and is usually significantly so; (3) the absolute sizes of the CC and its divisions tend to be the same for males and females, and not significantly larger for males, whatever their brain weights. This latter pattern is very different from most other parts of the CNS which have been volumetrically measured (see below). (4) There are significant but not large correlations between brain size and CC variables, except for SPLNDV (not significant), which probably is best explained by its high degree of sexual dimorphism.

#### Other Neural Structures and Sexual Dimorphism

As mentioned earlier, the patterning of the statistics for the CC is different from that for other CNS structures, aside from the hypothalamus (e.g., Swaab and Fliers, 1985). Consider the data presented by Wessely (1970) for the rhombencephalon, cerebellum, and brain weight. As in all samples encountered thus far, male brain size is significantly larger than female brain size (see table 69.9) as are the volumes for the cerebellum, rhombencephalon, and ventricles. When expressed as a percentage of brain weight, however, the ratio of differences between males and females becomes insignificant.

Table 69.10, based on the data of Klekamp et al. (1987, 1989), shows that for very small and large struc-

tures the male brain is significantly larger than in females. When corrected for brain size, however, these values are not significantly different. In other words, the statistical patternings of male-female sexual dimorphism are different and opposite for the CC: The absolute values do not differ significantly, but the ratios do.

Another example of this statistical patterning can be seen from the study done by Zilles (1972), who published data for a large sample of human brains (35 males, 43 females) for brain weight, cortex weight, and corpus callosum cross-sectional area (table 69.11). At the time that Zilles' paper was written, there was no controversy regarding sexual dimorphism of the corpus callosum, and Zilles' data show that, while the absolute value of the female CC area is greater than in males, it is not significantly so. Using Zilles' data we show that it is significantly greater in females than in males when corrected for brain weight. The absolute values for brain and cortex weight are much larger in males, with a very high degree of statistical significance ( $t$  values  $> 5.0$ ) compared to either the CC area or relative measures.

Many of the studies cited earlier (Appendix I) which claim to find no significant CC size difference between males and females do not permit similar testing. For example, Clarke et al. (1989: table 69.3, p. 226, show that in their MRI sample of brains (5 males, 7 females) the female CC area was 550 mm<sup>2</sup> while the male area was 540 mm<sup>2</sup>, without any significant difference. This is consistent with our findings. But one would expect the brain size differences to be highly significant, and thus the relative size of the female CC might be significantly larger relative to the brain than in males. Their postmortem figures, however, show an extraordinary difference between males and females (male = 680 mm<sup>2</sup>, females = 590 mm<sup>2</sup>), which is significantly larger in males at the 0.005 level. This is a very unusual finding, as the remainder of their table 69.3 demonstrates, since no other published studies show such a striking difference, and this is completely at odds with their M.R.I. sample.

Incidentally, Clarke et al. (1989: p. 226) confused the CC area values from the Byne et al. (1988) MRI study. The 602 and 583 mm<sup>2</sup> values for males and females in the Byne et al. study are only for those under 40 years of age. The 15 and 22 numbers refer to all ages, and the average CC area is 519 and 601 mm<sup>2</sup>, respectively, once again showing females to be larger in absolute size. This is another example of consistency with our findings, even though the authors strongly disagree with our earlier conclusions. Without brain weights, the relative size cannot be known. Similarly, the posterior one-fifth area is given as 170 and 160 mm<sup>2</sup>, respectively, for females and males by Byne et al. (1988), showing that females have a larger absolute splenial

size, again consistent with our findings. These differences may not predict gender according to the methods used by Byne et al. (1988), but they are certainly different from what one finds in the rest of the brain when looking for sexual dimorphism.

Correspondingly, the relatively large MRI sample, reported by Habib et al. (1991) to show no significant differences in CC area or morphology between males and females, does not provide adequate brain size corrections. (Correcting for size by merely measuring A-P distance of the whole brain slice is not the same as weighing the brain, and hardly a true correction for brain size,<sup>2</sup> considering the wide variation in cranial shape that exists in all populations.) The male sample is 35 cases, whereas the females number 18. As male brain sizes are usually significantly larger than females, might it not be possible that a larger sample of females would show a higher average value but with similar degrees of overlap? The 4.2% difference between male and female CC areas is much less than the usual 9–12% difference in brain size. Again, the differences between values between CC areas for males and females are much less than for the usual brain size differences.

Lastly, it is interesting to examine the Demeter et al. (1988) sample. Maximal splenial width was 11.8 mm for males and 11.6 for females, and thus nearly equal; the posterior one-fifth of the CC was 165 mm<sup>2</sup> for both males and females, yet total CC area was 627 and 582 mm<sup>2</sup> for males and females (hardly inconsistent with our findings). These authors did not test for relative values, however, even though they had measured brain weights. The differences between brain weight for males and females are extraordinarily large in this sample, with five (out of 21) of the males having brain weights greater than 1,500 grams (one value is greater than 1,700 g), while the highest female brain weight appears to be about 1,400 g, with at least four female values under 1,200 g. The mean brain weight is not reported for either sex, but the two means must differ at a very high level of statistical significance. The weak trend of a correlation between CC area and brain weight reported by the authors could be due to the relatively large size of the female CC or to the large sample difference of the mean brain weight between males and females. The brain weights in deLacoste-Utamsing and Holloway's (1982) study differed from that in the Holloway and de Lacoste (1986) study in that, in the second sample, female and male brain weights were almost equal (1,202–1,248 grams). In the first study the weights were 1,205 and 1,379 g, respectively.<sup>3</sup>

In sum, and as shown by Appendix I, almost every study that claimed no significant sexual dimorphism in the CC or its divisions has not studied the *relative* size of the callosum or the splenial portion. In fact, almost every study cited shows data that are consistent with

our findings regarding a high degree of dimorphism in brain weight, but either equality or larger CC dimensions for the female corpus callosum, particularly in the splenial portion.

The question of absolute vs. relative size of the CC in human males and females will require far larger autopsy samples than have been published thus far, or where there are adequate brain size controls for a large sample of MRI data. In the meantime, the three new autopsy samples presented in this report indicate that there is a congruent pattern of statistical findings which show that in samples where the male brain is significantly larger than those of females, the CC area is either the same absolute size or slightly larger in females, and that relative to brain size, the female CC appears to be significantly larger than that of males. These findings were obtained even after reducing the samples by removing both the lowest and highest value of the CCAREA for one male and female each. As far as we can determine, this trial was never attempted on the other studies. Small samples can be very sensitive to extreme values, as our trials indicated.

The posterior part of the CC (meaning the splenium as measured by the posterior fifth of CC area and dorsoventral splenial width), also shows strong size dimorphism, suggesting (but certainly not proving) that those areas of the cortex, such as the inferior and superior parietal lobules and the inferior temporal lobe, might have relatively more interconnections through the CC in females than in males. These apply only to averages, as the range of overlap is very great. Lastly, in this context we cite the studies of Hines et al. (1992), who appear to show results that tie together size differences in the splenial portion of the CC for females and a range of cognitive tests implicating the above cortical areas.

As interesting as the functional implications of these studies might be, it is important to stress the high degree of overlap of female and male values, and also to stress that larger sample sizes and studies which relate CC size variation to behavioral variation are necessary (which only Hines et al., 1992, have done). In addition, more microscopic analyses of fiber counts, diameters, and the ratio of myelinated to nonmyelinated fibers would be essential to better understand the nature and degree of the dimorphism (e.g., Aboitiz et al. 1992; Wium, 1984).

Comparative studies (e.g., de Lacoste and Woodward, 1988; Heilbroner and Holloway, 1989; Holloway and Heilbroner, 1992) suggest that the kind of dimorphism reported herein is absent in New and Old World monkeys. The deLacoste and Woodward (1988) study claims such a dimorphism for the pongids and strepsirrhines; however, the data need amplification, as the number of species studied was large but the sample

sizes within each were very small. Their study cannot rule out the possibility that human sexual dimorphism of the CC as reported here is species specific in the human, although dimorphism of the total size of the CC does exist in some rodents (see Deneberg et al., 1991). If the dimorphism is species specific, it raises the interesting question of how it got to be that way. Without a better understanding of the variation in the ultrastructure of the CC (meaning fiber components, myelinated and unmyelinated, diameters, etc.), we cannot answer that question. More quantitative study is clearly warranted and to be encouraged.

Finally, we note that these findings are in the main part consistent with earlier studies. We cannot explain each and every discrepancy, given the complexities of sample size and statistical analyses. MRI and cadaver-based studies are radically different methodologies, each with advantages and disadvantages. We emphasize the findings of *relative* (i.e., to the size of the brain) dimorphism favoring the CC splenial region in females. Most of the studies claiming to be at odds with our earliest findings focus either exclusively on *absolute* values or MRI samples where there are no adequate controls for brain size, and thus beg the question of *relative* size differences. Methodology, sample size, and perspective all play a role in these discrepancies. We can only note that when each sample is viewed in both absolute and relative terms (Appendix I), almost every one of them indicates near equality of the corpus callosum and its divisions between males and females, yet significantly higher brain weights in males. These facts are not contradictory to our findings, as has been claimed; rather, they are fully concordant with our observations and claims regarding *relative* sizes of the CC. We believe that the apparent inconsistencies are a function of failing to carefully control for brain size. As far as our earlier studies are concerned, we believe that the differences between this study and our earliest ones are best explained by sampling factors.

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### Notes

1. The purpose of this paper is to provide a new data rather than speculate about the reason for the findings. Anonymous reviewers ap-

pear disappointed that such speculation isn't reported here. One of us (RLH) does believe that there exists sexual dimorphism (with overlap) in cognition and that both the cerebral cortex and its interconnecting bundle, the corpus callosum, contribute to that dimorphism as well as cultural programming. The evolutionary change from a hominoid with relatively shorter periods of infant and child dependence to hominids with greater degrees of such dependence represents a dramatic evolutionary option. Another of us (RLH) believes a complementary strategy, involving greater degrees of behavioral specialization among males and females, was useful for that important adaptation, which would provide the basis for human brain reorganization and size increase. RLH does believe females are more socially competent in communicative skills, that males are better at visuospatial tasks involving directionality and placement in complex space, and that these two basic moieties of cognitive adaptations made for a more successful hominid adaptation for survival, particularly for animals with offspring dependent on physical and social nurturance for longer postnatal periods and extended growth durations of the brain.

2. Habib et al. (1991) state: "[A]nd each picture was magnified to a standard size. In order to control for brain size, magnification rates of MRI midsagittal sections were varied among individuals so as to reach a *standard brain anterior-posterior axis of 170 mm for each case.*" (p. 46, emphasis mine). This is not the same as controlling for brain size, given the high degree of variability in brain and cranial shapes in humans. This technique is essentially equating one measurement on a midsagittal section with brain size. No one can reliably predict brain weight with less than three dimensions. Most of the other MRI studies cited in this paper do not even use this weak method of controlling for size.

3. One of us (RLH) would like to point out that neither deLacoste or myself were ever happy with the small samples that were reported in 1982 and 1986. Both of us regarded our findings as preliminary and thought that the absolute difference in splenial width would probably vanish with larger samples. Our values, as it turns out, are not typical. This sample, which was essentially completed in 1987, could not be reported until now due to illness of the senior author. We furthermore maintain that it is the relative size of the corpus callosum that is sexually dimorphic. All of the studies cited, except our own, have focused mostly on absolute differences, and not one has looked at ratio data by carefully examining brain size. ANOVA and ANCOVA techniques do not adequately test our basic hypothesis that the CC and some of its components are relatively larger in female human brains. Instead, they test the relative degree of variance explained by brain size and/or sex, depending on the investigators' goals.

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#### Appendix I: A Review and Summary of All Studies Available on Sexual Dimorphism of the Corpus Callosum to Date

Each study, arranged chronologically, provides a summary of whether the sample was from autopsy or magnetic resonance imaging, the sample sizes when available, whether statistically significant sexual dimorphism was reported, and the mean CC area for males and females. Where available, other measures such as the splenium are given, as well as information regarding the use of brain size as a control. Lastly, we comment on the possibility of the results being similar to ours.

1. *de LaCoste-Utamsing and Holloway, 1982.* Autopsy data, 9 males, 5 females. Dimorphism significant: mean C areas = 704 (M), 708 (F). Relative CC measures reported. Both splenial thickness and posterior one-fifth dimensions higher in females, which have not been replicated. Given small sample size, the results for absolute differences have not been duplicated.
2. *\*Wium, 1984.* Autopsy data, but not given. Reported in the abstract that females had absolutely larger splenia than males, while males had more fibers in the splenial portion. Cannot be certain without the data, but the direction of splenial size is consistent with our findings.
3. *Bell and Variend, 1985.* Autopsy data, 40 children. Dimorphism not significant. Brain weight not studied. Children aged from birth to 14 years, giving very small sample sizes within age groups. Not truly relevant to our studies.
4. *\*Witelson, 1985.* Autopsy data, 12 males, 30 females. Dimorphism not significant. Mean CC area, 672 (M), 654 (F). Relative measures were not given. Neither consistent right-handers (CRH) or mixed-handers (nCRH) showed any sexually dimorphic differences, except in brain size. Ratio data (not in study) using published averages show females have proportionately larger CC areas than males, as well as larger posterior fifths (splenium). Male brain weights were much larger than those of the females. This study could be consistent with our observations regarding the relative size of the CC.
5. *Bleier et al., 1986.* Magnetic resonance imaging. Dimorphism reported as not significant. Data not provided. No attempt was made to ascertain the relative size of the CC or its component parts.
6. *\*Holloway and deLaCoste, 1986.* Autopsy data, 8 males, 8 females; dimorphism significant. Mean CC area 618 (M), 744 (F). Brain size studied and relative CC sizes reported. Splenial width was 10.3 in males and 13.3 in females. Posterior one-fifth was not reported. Brain weight was not significant between males and females. Sample sizes very small, suggestive of outlying values for female CC area and maximum splenial width.
7. *\*Weber and Weis, 1986.* Autopsy data, 18 males, 18 females; dimorphism not significant. Mean CC area was 639.5 (M), 613 (F). Brain size was studied, averaging 1,029 in males and 890 in females, a significant dimorphism, but with unusually low weights. (Average age was 74.7, which is high.) The splenial posterior one-fifth area was 164.5 in males and 162.4 in females. The CC area was marginally higher in males, but S.D.s were in the vicinity of 105.3. Relative figures not provided. These results are consistent with our observations, given the near equality of CC area and splenial portion between the sexes.
8. *\*Yoshii et al., 1986.* Magnetic resonance imaging, 14 males, 19 females; dimorphism not significant. Data not provided. No significant differences were reported in the abstract, and brain weights were not studied. A “blinded rating” revealed the female splenium to be more bulbous ( $P = .025$ ). No significant handedness effect. Possibly consistent with our findings.
9. *\*Kertesz et al., 1987.* Magnetic resonance imaging, 51 males, 53 females; dimorphism not significant. Mean CC area was 724 (M), 716 (F). Axial brain size correction (horizontal) shows female ratio is significantly larger than male ratio. Sagittal section size correction larger in females but not significantly so. Splenium to genu size ratios

were larger in 85 cases for females and 78 cases for males. Chi-square showed no significant difference. Possibly consistent with our observations on relative CC size.

10. *\*Oppenheim et al., 1987.* Magnetic resonance imaging, 40 males, 40 females; dimorphism not significant. Data not given. Mean splenial area was reported as a percent of total CC area, and was higher in females (31.2% females, 30.4% males). Splenial width was 23.7% in males and 24.1% in females. Brain size was not studied. Total CC areas not given. The percentage figure may not be statistically significant, but the direction clearly favors females and is consistent with our views regarding relative size.
11. *\*Byne et al., 1988.* Magnetic resonance imaging, 15 males, 22 females; dimorphism reported not significant. Mean CC area was 519 (M), 601 (F). Brain size was not studied. Both the CCAREA and posterior one-fifth (splenium) were absolutely larger in females. The splenium was 160 mm<sup>2</sup> for men and 168 mm<sup>2</sup> for women in the age > 40 sample. In the total sample, posterior one-fifth was 170 in females and 160 in males. Given these findings, and the usual dimorphism of brain size being larger in males, these results are fully consistent with our findings.
12. *\*Demeter et al., 1988.* Autopsy data, 22 males, 12 females; dimorphism not significant. Mean CC area was 627 (M), 582 (F). Relative measures not given. Posterior one-fifth area (splenium) was 165 mm<sup>2</sup> in both males and in females. Splenial width was 11.8 mm in males and 11.6 mm in females. Brain weights for males cluster between 1,300 and 1,700 cc. Female brain weights cluster between 1,050 and 1,200 cc, and do not overlap male values. Six male values are above 1,500 cc. With these large brain size differences but relatively minor differences in callosal sizes, these results could well be consistent with our observations.
13. *O’Kusky et al., 1988.* Magnetic resonance imaging, 26 males, 24 females. No significant dimorphism. Brain size was not controlled nor measured. Values of total CC area or thicknesses and regions of the CC not given for males and females, respectively. The averages are mixed sexes comparing epileptic patients and normal controls for different handedness groups. No significant differences reported. Without sex values, no comparisons can be made.
14. *\*Weis et al., 1988.* Magnetic resonance imaging, 20 males, 26 females; dimorphism not significant. Mean CC area was 669.9 (M), 665.2 (F). Total CC areas are almost equal for males and females. The splenial (posterior one-fifth area) was 191.5 for males, but 199.9 for females. Brain size was not considered. This study could be consistent with our findings, particularly if brain size was dimorphic.
15. *\*Clarke et al., 1989.* Both autopsy and magnetic resonance imaging data collected. Mean CC area was 680 (M), 590 (F). Relative measures not reported. In autopsied sample, males had significantly larger CC areas than females (680 mm<sup>2</sup> vs. 590 mm<sup>2</sup>). In the MRI sample, females had a slightly larger CC area (females = 550 mm<sup>2</sup> and males = 540 mm<sup>2</sup>; not significant). Posterior one-fifth (splenium) larger in MRI women (152–148), but in autopsied sample the male mean was larger (173–165). In both samples the females had a higher relative area of the posterior one-fifth splenial region. Maximum splenial widths only slightly larger in males in both samples (11.1 vs. 10.7; 10.8 vs. 10.3 in autopsy and MRI samples, respectively). Bulbosity index significantly higher in females for autopsy group, but larger in males for MRI sample. The MRI samples were small ( $N = 5$ , males,  $N = 7$ , females). Since brain weights are not reported, it is impossible to ascertain whether the relative sizes were different between the sexes. This study offers mixed results, some of which are consistent with our study, for example, in the splenial region.
16. *\*Witelson, 1989.* Autopsy data. Dimorphism reported as significant. Mean CC area was 674.5 (M), 650.4 (F). Patients were terminal cancer victims. Brain weights available, but only cerebrum weight was used. Relative data not provided. Report shows that regions were “corrected” for cerebrum weight by using two-factor (hand and sex) ANCOVA methods exclusively. The total CC area was larger in males and “proportional to overall brain size” (p. 825). The female sample was almost three times as large as the male sample for CRH. The isthmus was larger in females in both handedness groups. These results are mixed, but essentially provide evidence for some sexual dimorphism in the presplenial section, the so-called isthmus. Witelson’s characterization of other studies is faulty in that

no relative studies were done. These results are thus only partially consistent with our study regarding shape and relative size.

17. \**Elster et al., 1990.* Magnetic resonance imaging, 60 males, 60 females. Dimorphism mixed. Mean CC area was 719 (M), 692 (F). Cerebral area in midsagittal plane was used as brain size control. Ratio data show some significant differences favoring females as in splenial width/CC length and CC area/cerebral area. Most areal and linear measures were not significantly larger in males. These results do tend to show a small but persistent dimorphism favoring the female, and thus support some of our results.

18. \**Going and Dixon, 1990.* Autopsy data, 17 males, 16 females; dimorphism not significant. Mean CC area was 656 (M), 621 (F). Cerebrum weight was available, but not used directly for ratios. Posterior one-fifth area was 192 mm<sup>2</sup> in males and 170 mm<sup>2</sup> in females. "Correcting" for cerebral size gave males a CC area of 631 mm<sup>2</sup> and females 646 mm<sup>2</sup>. None of the authors' statistics showed any significant dimorphism between males and females. The method of size control is faulty when an average correction is used for all brains rather than individually, and the averages resulting from dividing CC area by cerebral weight show females with a ratio of 1.305 and males with an average of 1.224. The average age of females was 82 years, that of males was 74.4 years. These are very high, and given the loss of neurons with advancing age, makes the female sample particularly suspect. The S.D.s of the CC areas are about 20%. Maximum width of the splenium was reported to have been measured but no figures are provided. This study claims not to be in support of our results, but the methods do not permit a true comparison. Some of the averages still favor the females, whether significant or not.

19. \**Holloway, 1990.* Autopsy data, 13 males, 9 females; dimorphism significant; mean CC area was 705 (M), 765 (F). Cortical area, but not brain weight, were studied. Relative measures were significantly larger in females, as was maximum dorsoventral splenial width. Total cortical area not significantly different between males and females.

20. \**Prokop et al., 1990.* Autopsy and magnetic resonance imaging. We have been able to secure only an abstract of this paper. Not certain of ages, nor whether any ratio data were studied. The abstract claims there were no significant differences or sexual dimorphism.

21. \**Allen et al., 1991.* Magnetic resonance imaging, adult and children (122 age-matched adults, 24 age-matched children). Dimorphism was not significant in absolute measures. Mean CC area was 6.87 cm<sup>2</sup> in males and 6.80 in females. Brain size was controlled by measuring the area of the cerebral cortex in midsagittal section, and was significantly higher in males (92.96 vs. 88.57 in females). The maximum splenial width was 1.27 in males and 1.35 in females, the latter being significant at the .035 level. A "bulbosity index" was clearly higher in females and significantly so. CC sizes are almost equal between the sexes, while the splenial width is larger in females. At the same time, the area of cerebral cortex is significantly higher in males. These results, while mixed, are basically consistent with our study.

22. \**Deneberg et al., 1991.* Magnetic resonance imaging, 51 males, 53 females; dimorphism not significant. Mean cc area was 731 (M), 722 (F). Brain size was an area calculated from one horizontal section as per Kertesz et al. (1987), and thus not a strict control of brain size. Factor analysis was performed on 99 width measures to extract a smaller set of measurements. In the region where maximum splenial width occurs, the female width (right-handed) was 11.59 mm, the male was 11.625 mm. For left-handers, the female sample mean was 11.4 and 11.53 for males. Sex effects varied depending where the width measures occurred, being larger for females in some cases and smaller in others. In no case were ratio data tested, nor does the paper provide any discussion of the supposed brain size control, that is, one horizontal brain area. Without this latter measure we cannot assess the dimorphism in approximate brain size, or relative sizes of the CC components. We cannot be certain whether these results are or are not consistent with our observations.

23. \**Habib et al., 1991.* Magnetic resonance imaging, 35 males, 18 females; dimorphism not significant. Mean CC area was 809 (M), 775 (F). Authors claim that brain size was controlled by adjusting CC length to a standard sagittal length of 170 mm. This cannot truly control for brain size. Posterior subregions are all absolutely higher in males but not significantly so. (P1 88.8 M, 86.9 F; P2 86.01 M,

83.7 F; P3 219.9 M, 201.4 F) Males and female values are quite close, but without true brain size, the relative size of the CC components cannot be ascertained. These results are not truly consistent with our study. However, when handedness is examined, consistent right-handed (CRH) males had a total CC area of 746.5 mm<sup>2</sup> and females were 770.6. The P1 and P2 subregions were also larger in females: P1 101.4 (F) to 81.8 (M); P2 92.2 (F) to 77.06 (M). P3 larger in males for either handedness group. For nonconsistent right-handers, males were larger than females in all CC measures. These results are thus mixed, partly consistent with our observations and partly not.

24. \**Witelson and Goldsmith, 1991.* Autopsy data, 8 males, only. Nonconsistent right-handers (nNCRH) had larger CC areas and isthmus region than CRH males. Brain weight was studied, but relative values not reported. These values are consistent with Witelson's earlier reports.

25. \**Steinmetz et al., 1992.* Magnetic resonance imaging, 26 males, 26 females; the statistical significance of dimorphism was mixed, but not in mean CC area. Mean CC area was 678 (F), 673 (M). Brain size not controlled. No significant differences in most CC regions, except for 2 out of 3 posterior regions, which were absolutely larger in females than in men. The percentages of these posterior areas to the total CC area were significantly larger in females in two posterior regions. The differences held for sex but not handedness. These results are clearly consistent with our observations.

## Summary

Sixteen out of 25 studies (not including this or our earlier studies) that claim to show no significant sexual morphism of the corpus callosum have some results that are consistent with our findings when the relative size of the corpus callosum is considered. Such studies are prefixed by an asterisk (\*) to suggest that more data, particularly of a relative nature, might corroborate some of our findings regarding shape and relative size.



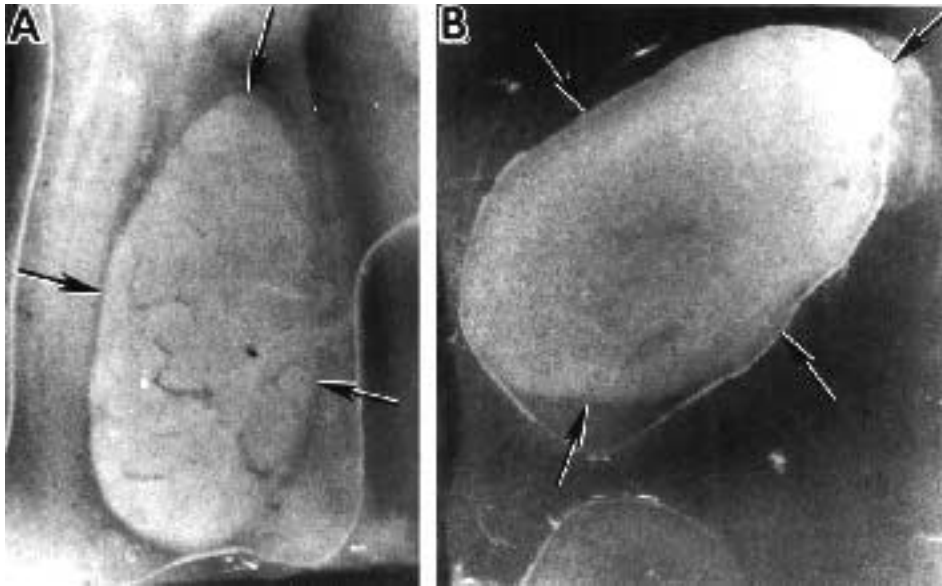
Although it has long been recognized that the brain influences sex differences in animal behavior and reproductive physiology, neuroanatomical sexual dimorphisms have only recently been discovered that may underlie these functional differences. In regions of the brain known to control sexually dimorphic function, there are intracellular and ultrastructural neuroanatomical sex differences in the size of nerve cell nuclei (Pfaff, '66; Dörner and Staudt, '68); dendritic branching patterns of neurons in the preoptic area (POA) in the rat (Hammer and Jacobson, '84) hamster (Greenough et al., '77), and the macaque monkey (Ayoub et al., '83); and synaptic organization of the POA (Raisman and Field, '71), arcuate nucleus (Matsumoto and Arai, '81), and medial amygdala (Nishizuka and Arai, '81) of the rat. In contrast to these intracellular and ultrastructural differences, there are dimorphisms in terms of the volume of nuclei involved in vocal communication in songbirds (Nottebohm and Arnold, '76), in the bed nucleus of the stria terminalis in guinea pigs (Hines et al., '85) and rats (del Abril et al., '87), and in the POA of rats (Gorski et al., '78), gerbils (Yahr and Commins, '82), guinea pigs (Hines et al., '85), ferrets (Tobet et al., '86), and quail (Panzica et al., '87). In addition, scientists have recently identified structural sex differences in regions not directly related to reproductive function: in rats, there may be sexually dimorphic patterns of cortical and hippocampal asymmetries (Diamond et al., '83); and the corpus callosum is sexually dimorphic in terms of midsagittal area (Berrebi et al., '88), the number of axons, and extent of myelination (Juraska and Kopicik, '88).

Although numerous reports now exist on sex differences in laboratory animals, relatively little is known about sex differences in the human brain. In the preoptic-anterior hypothalamic area, a region of the brain implicated in rodents and subhuman primates in gonadotropin release (Gorski, '68; Plant et al., '79; Pohl and Knobil, '82), sexual behavior (Arendash and Gorski, '83; Oomura et al., '83), and maternal behavior (Jacobson et al., '80), there are dramatic sex differences in humans in the volume of several nuclei (Swaab

and Fliers, '85; Allen et al., '89); similarly, a region of the bed nucleus of the stria terminalis is larger in men than in women (Allen and Gorski, '90); the shape of the suprachiasmatic nucleus differs between men and women (Swaab et al., '85); and Onuf's nucleus in the spinal cord, which innervates the perineal muscles, contains more motoneurons in men than in women (Forger and Breedlove, '86). In addition, several neurally controlled functions not directly related to reproduction are also sexually dimorphic: there are subtle differences in terms of cognitive abilities whereby women generally score better on tests of verbal abilities, and men on exams of mathematical skills (Harris, '78; Kimura, '87); there is a prevalence in boys relative to girls of several language disorders, including dyslexia, delayed speech acquisition, and stuttering (Hier, '79); and there is greater functional asymmetry in the male than in the female brain (McGlone, '80; Beaton, '85; Kimura, '87).

Recent evidence suggests that there may be a neuroanatomical basis for these functional differences. The massa intermedia (MI) of the thalamus is more often present in women than in men (Morel, '48; Rabl, '58; Davie and Baldwin, '67; Samra and Casper, '68), the direction of the asymmetry of the temporal planum differs between male and female fetuses (Wada, '75), and the shape of the splenium of the corpus callosum (CC) may be more bulbous in females (de Lacoste-Utamsing and Holloway, '82; Allen et al., '91). The latter observation has led to speculation that the axonal connectivity between the two hemispheres of the brain differs between the sexes; however, subsequent studies of the CC have produced conflicting results (for review see Allen et al., '91) in part because a specific, regional sex difference in this heterogeneous structure may be difficult to isolate by arbitrarily partitioning and measuring parts of the CC based on its shape alone.

Should there be a general sex difference in the connectivity between the two cerebral hemispheres, either in terms of axons and/or other neural components such as glia, myelin, and connective tissue, then other structures present at the midsagittal plane of the brain



**Figure 70.1**

*A* and *B* are photographs of the midsagittal region of the anterior commissure and the massa intermedia, respectively. The *arrows* indicate the borders of the anterior commissure and the massa intermedia.

may also be sexually dimorphic. In contrast to the CC, two smaller structures, the anterior commissure (AC) and the MI, connect relatively specific regions of the brain. Therefore, a sex difference in either structure may become apparent on examination of the total midsagittal area without further arbitrary partitioning.

#### Materials and Methods

The brains used in this study, which were obtained from two Southern California hospitals, had been removed within 24 hours postmortem and placed directly into acetatebuffered 10% formalin for 2 to 4 weeks prior to coronal sectioning performed during routine autopsy. Following autopsies, the present investigators collected approximately 500 samples of brain tissue containing either the midsagittal region of the AC or the medial thalamic nuclei and the MI, if present; however, a note was made if no MI was present. These samples were collected without knowledge of gender. If one of the regions was removed or damaged during autopsy, only the other structure was collected from a given individual. Subsequently, samples were eliminated if they were from an individual whose medical record indicated neuropathology, neuroendocrine disorder, or homosexual orientation. This resulted in 138 ACs and 156 MIs (inclusive of subjects without an MI) whose identification codes were organized into four columns based on structure and gender, and ordered according to age. We age-matched subjects to obtain 50 pairs of males and females for both ACs

and MIs. The region of the brain containing the medial area of the AC and the MI were sectioned in the midsagittal plane, and this surface of each structure was placed against a glass, adjacent to a ruler taped at the same plane, photographed, and made into slides (figure 70.1a,b). The slides of the ACs and MIs were projected onto white paper at a magnification of 20 $\times$  and 12 $\times$ , respectively.

Without knowledge of age or gender, two individuals traced each image. The area of each outline was determined by using a Bioquant Hipad digitizer, which is adjusted to correct for magnification (Bioquant IBM Program version 2.1; R & M Biometrics). Each measurement of the two investigators was compared, and if there was greater than a 5% difference between the areas of the tracings, the structure was reexamined by a third investigator and the two measurements in closest agreement were averaged.

The paired t-test was used to compare the areas and brain weights of the AC and the MI between male and female subjects. However, when the area of the MI was examined in only subjects with an MI, the independent t-test was used. Sex by age interactions was tested, using the paired paradigm, by regressing the difference in area between each male and female pair with the average age of each pair (Smith and Choi, '82). Pearson's correlation coefficient (Dawson-Saunders and Trapp, '90) was used to test for correlations between the measurements of the two tracers, brain weight and area, age and area, and between areas of the AC and MI when both were examined within a given individual.

**Table 70.1**Mean  $\pm$  S.E.M. for age, brain weight, and area of the anterior commissure (AC) and massa intermedia (MI)

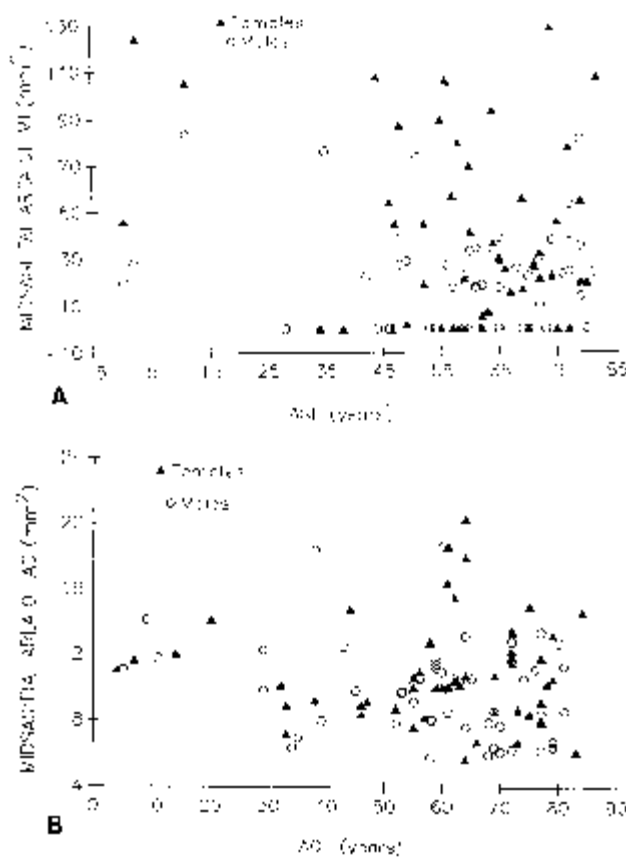
	<i>N</i>	Age (Years)	Brain Weight (Grams)	Area (mm <sup>2</sup> )	<i>p</i>
AC—all females	50	58.38 $\pm$ 2.7	1,249 $\pm$ 15	11.00 $\pm$ 0.45	0.038
AC—all males	50	58.52 $\pm$ 2.7	1,332 $\pm$ 16	09.84 $\pm$ 0.41	
AC—adult females	47	61.57 $\pm$ 2.14	1,257 $\pm$ 15	10.96 $\pm$ 0.48	0.030
AC—adult males	47	61.72 $\pm$ 2.09	1,334 $\pm$ 17	09.68 $\pm$ 0.43	
MI—all females w/MI	39	59.35 $\pm$ 3.04	1,233 $\pm$ 17	50.31 $\pm$ 5.8	0.011
MI—all males w/MI	34	58.92 $\pm$ 3.55	1,290 $\pm$ 36	32.83 $\pm$ 3.2	
MI—adult females w/MI	36	63.92 $\pm$ 1.76	1,238 $\pm$ 16	46.88 $\pm$ 5.67	0.023
MI—adult males w/MI	31	64.19 $\pm$ 2.15	1,327 $\pm$ 22	31.77 $\pm$ 3.12	
MI—all females	50	59.53 $\pm$ 2.54	1,236 $\pm$ 15	39.26 $\pm$ 5.4	0.009
MI—all males	50	59.24 $\pm$ 2.63	1,307 $\pm$ 26	22.33 $\pm$ 3.1	
MI—adult females	47	63.04 $\pm$ 1.6	1,240 $\pm$ 15	35.91 $\pm$ 5.2	0.024
MI—adult males	47	62.75 $\pm$ 1.8	1,333 $\pm$ 17	20.96 $\pm$ 3.0	

## Results

### Anterior Commissure

There was a highly significant correlation between the two measurements of the two individual tracers ( $r = 0.93$ ;  $p < 0.0001$ ). Three pairs of children ranged from 4 to 14 years of age and 47 pairs of adults ranged from 20 to 84 years, with a maximum of 10 years and an average of 2.2 years between pairs (table 70.1, figure 70.2a). The midsagittal surface area of the AC of the female subjects (mean  $\pm$  S.E.M. =  $11.003 \pm 0.45$  mm<sup>2</sup>) was an average of  $1.165$  mm<sup>2</sup> or 11.8% greater than that of male subjects ( $9.838 \pm 0.41$  mm<sup>2</sup>) (paired  $t$ -test:  $p = 0.038$ ), despite the fact that the male brains ( $1332.83 \pm 16.41$  gms) were 6.7% larger than those of the females ( $1248.7 \pm 15.48$  gms) ( $p = 0.0006$ ). Similarly, the midsagittal surface area of the AC was greater in women ( $10.964$  mm<sup>2</sup>) than in men ( $9.76$  mm<sup>2</sup>) ( $p = 0.030$ ); however, there was an insufficient number of children to determine whether there was a sex difference during childhood. There was no significant correlation between brain weight and size of the AC ( $r = 0.13$ ;  $p = 0.186$ ). In adults, the area of the AC did not change with advancing age ( $r = 0.04$ ;  $p = 0.69$ ), for women ( $r = 0.05$ ;  $p = 0.718$ ), or men ( $r = 0.15$ ;  $p = 0.31$ ).

Since visual inspection of figure 70.2a suggested that the difference in area between males and females might be greater after the age of about 50, we tested our data for a sex by age interaction by regressing the difference in area between males and females of each pair (in this case, the male value minus the female value) against the average age of each pair ( $r = 0.209$ ;  $p = 0.145$ ) (Smith and Choi, '82). However, at the correlation coefficient of  $r = 0.209$ , a total of 200 pairs would be necessary to detect a significant ( $p = 0.05$ ) sex by age interaction at a power of 0.81.

**Figure 70.2**

(a) Anterior commissure (AC) midsagittal cross-sectional area as a function of age. In adults, there was no significant change in the area of the AC ( $r = 0.04$ ;  $p = 0.692$ ) in women ( $r = 0.054$ ;  $p = 0.717$ ) or in men ( $r = 0.152$ ;  $p = 0.308$ ). (b) Massa intermedia (MI) midsagittal cross-sectional area as a function of age. In adults, there was no significant change in the area of the MI ( $r = 0.0196$ ;  $p = 0.851$ ) in women ( $r = 0.007$ ;  $p = 0.962$ ) or in men ( $r = 0.035$ ;  $p = 0.815$ ).

There was no correlation between the area of the AC and duration in fixative ( $r = 0.2$ ;  $p = 0.87$ ); furthermore, the average duration in fixative was 20.3 months for females and 20.1 months for males ( $p = 0.90$ ).

#### Massa Intermedia

There was a highly significant correlation between the two measurements of the two tracers for only subjects *with* an MI ( $r = 0.87$ ;  $p < 0.0001$ ) and for *all* subjects ( $r = 0.91$ ;  $p < 0.0001$ ). Three pairs of children ranged from 0.2 to 11 years of age; 47 pairs of adults ranged from 28–82 years, with a maximum of 6 years and an average of 1.1 years between pairs (table 70.1, figure 70.2b). The MI is more frequently absent in males than in females (Morel, '48; Rabl, '58; Davie and Baldwin, '67; Samra and Cooper, '68). However, in this study, the sex difference in the midsagittal area of the MI was not a result of its greater absence in males than in females (32% of the males and 22% of the females had no MI), since the midsagittal surface area of the MI for subjects with an MI was greater in females ( $50.33 \pm 5.77 \text{ mm}^2$ ) by an average of  $17.5 \text{ mm}^2$  or 53.3% greater than in males ( $32.83 \pm 3.2 \text{ mm}^2$ ) ( $p = 0.011$ ; independent  $t$ -test), despite the fact that the male brain ( $1,290 \pm 36 \text{ gms}$ ) in these subjects weighed an average of 4.6% more than the female brain ( $1,233 \pm 17 \text{ gms}$ ) ( $p = 0.15$ ). Among adults with an MI the mean midsagittal surface area of the MI of women ( $46.88 \pm 5.7 \text{ mm}^2$ ) was  $15.11 \text{ mm}^2$  or 47.6% greater than that of men ( $31.77 \pm 3.1 \text{ mm}^2$ ) ( $p = 0.023$ ; independent  $t$ -test); however, there were only six children, thereby precluding statistical analysis on these subjects alone.

Among *all* subjects, the mean midsagittal surface area of the MI of females ( $39.258 \pm 5.39 \text{ mm}^2$ ) was an average of  $17.498 \text{ mm}^2$  or 75.8% greater than of males ( $22.33 \pm 3.1 \text{ mm}^2$ ) (paired  $t$ -test:  $p = 0.009$ ) despite the fact that the male brain ( $1,307 \pm 26 \text{ gms}$ ) weighed an average of 5.7% more than the female brain ( $1,236 \pm 15 \text{ gms}$ ) ( $p = 0.007$ ). Similarly, among adults the midsagittal surface area of the MI was greater in women ( $35.911 \pm 5.39 \text{ mm}^2$ ) than in men ( $20.956 \pm 3.10 \text{ mm}^2$ ) ( $p = 0.024$ ); however, there were only three pairs of children, which precluded statistical analysis of these subjects. There was no significant correlation between brain weight and size of the MI among subjects with an MI ( $r = 0.021$ ;  $p = 0.86$ ) or among all subjects ( $r = 0.058$ ;  $p = 0.562$ ).

In adults *with* an MI, the area did not change with advancing age ( $r = 0.116$ ;  $p = 0.352$ ) in women ( $r = 0.107$ ;  $p = 0.535$ ) or in men ( $r = 0.161$ ;  $p = 0.387$ ). Similarly, among *all* adults, the area of the MI did not change with advancing age ( $r = 0.02$ ;  $p = 0.85$ ) in women ( $r = 0.007$ ;  $p = 0.96$ ) or in men ( $r = .04$ ;  $p = 0.815$ ). There was no sex by age interaction for

either pairs where *both subjects had an MI* ( $r = 0.018$ ;  $p = 0.905$ ), or for *all* adults ( $r = 0.094$ ;  $p = 0.586$ ) when we regressed the difference in area between males and females of each pair (in this case, male value minus female value) with the average age of each pair.

The average duration in fixative for subjects with an MI was 18.8 months for females and 19.1 months for males, with no correlation between duration in fixative and area of the MI ( $r = 0.107$ ;  $p = 0.334$ ). For *all* subjects, the average duration in fixative was 18.5 months for females and 18.6 months for males, with no correlation between duration in fixative and area of the MI ( $r = 0.11$ ;  $p = 0.51$ ).

#### Relation between the Anterior Commissure and the Massa Intermedia

In 39 subjects in which both the AC and MI were obtained, there was no correlation between midsagittal surface areas of the AC and the MI ( $r = .047$ ;  $p = 0.803$ ). However, failure to obtain correlations could, in part reflect the great variability in areas of both the ACs and the MIs (table 70.1, figure 70.2a,b).

#### Discussion

The functional significance of sex differences in the AC and the MI is unknown. However, such sexual dimorphisms may underlie differences between males and females in terms of cognitive skills (Harris, '78; Kimura, '87), developmental language disorders (Hier, '79), and functional asymmetry (McGlone, '80; Beaton, '85; Kimura, '87). It is unclear whether these sex differences are due to differences in the number of axons, thickness of myelination, or, in the case of the MI, the number of cell bodies. However, in the rhesus monkey, the area of the AC does not correlate well with the number of axons (LaMantia and Rakic, '90). The size of the MI at the midsagittal surface may not relate to the interhemispheric axons or the cell bodies at the midsagittal plane; rather, it may underlie a sex difference in the volume, presence, and/or arrangement of nuclei which compose the thalamus.

#### Methodological Considerations

Although we matched our subjects for age, we did not match them for either the postmortem period prior to removing the brains from their skulls and placing them into fixative, or for the duration in fixative prior to histological preparation. Although either of these two variables could influence shrinkage, the postmortem period was less than 24 hours, and there was neither a significant difference in duration of fixation between male and female subjects, nor a significant correlation between duration of fixation and the midsagittal area of either the AC or the MI for either males,

females, or for all subjects combined. Furthermore, slight error could be introduced by imprecision of the midsagittal plane; however, all brains were sectioned through the third ventricle as close to the midline as possible. Although either of these variables may have introduced error into our measurements, it is unlikely that they contributed significantly to the sex difference. We believe that the 3.5-fold range in the areas of the ACs among all subjects and the 71.1-fold range in the areas of the MIs among subjects *with* an MI reflects the actual high degree of variation in the area of these structures within the human brain, rather than artifactual or methodological error; therefore, large sample sizes are necessary to demonstrate the presence of sex differences. Similarly, other investigators observed considerable variation in the area of the AC (Tomasch, '57; Demeter et al., '88) and the MI (Morel, '48; Rabl, '58; Davie and Baldwin, '67; Samra and Cooper, '68).

### Anatomy

**Anterior Commissure** The AC of the primate brain is a tract of axons that primarily connects the right and left temporal neocortices (Fox et al., '48; Whitlock and Nauta, '56; Pandya et al., '69; Pandya et al., '73; Rocha-Miranda et al., '75; Gross et al., '77; Jouandet and Gazzaniga, '79; Jouandet, '82). It is unknown whether sex differences in the area of the AC reflect differences in the number of axons, in myelination, connective tissue, or glia. In a small sample of human subjects, differences in the area of the AC reflected differences in the number of axons and not their density (Tomasch, '57). In contrast, in a study of the rhesus monkey, the midsagittal surface area of the AC did not correlate strongly with the number of axons ( $r^2 = .473$ ) (LaMantia and Rakic, '90). Although there may be axonal elimination due to atrophy with advancing age, in humans there is protracted myelination of the AC at least into adulthood (Yakovlev and Lecours, '67). Therefore, the relation between midsagittal area of the AC and the number of axons may actually change over the course of a lifespan, which in part can be accounted for by age-matching subjects should these two processes occur at a similar rate in both sexes.

**Massa Intermedia** The MI of the primate is composed of neurons and neuropil, as well as loosely organized axons that connect the thalami (Crouch and Thompson, '38; Glees and Wall, '48), and motor, premotor, and prefrontal areas (DeVito, '69; Künzle, '76). In humans, pro-somatostatin-derived-peptide-positive fiber tracts course through the MI (Bouras et al., '87). Neuroanatomists do not agree on which thalamic nuclei constitute the human MI, perhaps because of its considerable variability among individuals. However it

may be composed of nucleus rhomboideus (Sheps, '45), nucleus centralis medialis (Sheps, '45; Toncray and Krieg, '46), and/or nucleus reuniens (Rabl, '48). It is unknown whether variation among individuals in the midsagittal area of the MI reflect differences in neurons, glia, neuropil, connective tissue, or axons. In fact, sex differences in the MI may reflect differences in the size and/or presence or absence of the nuclei rhomboideus, centralis medialis, and/or reuniens, which are also present bilaterally.

### Sexual Differentiation

On the basis of our small sample of children, it is unclear *when* sexual differentiation occurs. However, genomic factors, the environment, and/or gonadal hormone levels may influence sex differences in the AC and/or MI. For example, structures reported to be sexually dimorphic in rats, including the cerebral cortex (Diamond, '88) and the CC (Berrebi et al., '88; Juraska and Kopcik, '88), are influenced by environmental factors both pre- and postnatally in a sexually dimorphic manner. The sexually dimorphic pattern of cerebral cortical asymmetry may be altered by both prenatal stress (Fleming et al., '86) and an enriched postnatal environment (Diamond, '88), and the CC in rats may be influenced prenatally by maternal alcohol consumption (Zimmerberg and Scalzi, '89) and postnatally by handling (Berrebi et al., '88), and each of these environmental factors influences males and females differently. More consistent, however, have been the observations in laboratory animals that all sexually dimorphic structures examined thus far have been shown to be influenced by perinatal gonadal hormone exposure. Therefore, based on animal studies, it is possible that environmental factors may influence the AC and MI in a sexually dimorphic manner, but perinatal gonadal hormones are also likely to be involved.

The mechanism by which environmental factors influence neural structure differently between males and females is unknown, although a gonadal hormone mechanism underlies the effect of prenatal stress in rats (Ward, '84). With respect to gonadal hormones, the elimination of neurons in sexually dimorphic nuclei appears to be determined by the presence or absence of gonadal hormones during a critical period of development (Nordeen et al., '85). Since there is uptake of gonadal hormones in the cerebral cortex of the developing rat (Sheridan et al., '74; MacLusky et al., '79) and rhesus monkey (MacLusky et al., '86) and an elimination of neurons and their axons during development (Berlucchi, '81), it is conceivable that gonadal hormones influence the number of axons and/or neurons of the AC and MI. Similarly, it is possible that (to some extent) sex differences in the AC and MI reflect the different influences of gonadal hormones upon

myelination: in rats, *estradiol* increases myelination (Curry and Heim, '66), and during development, 5- $\alpha$ -reductase, the enzyme that converts testosterone to dihydrotestosterone, is inversely related to myelination (Celotti et al., '87).

It is unknown whether the sex differences in the *presence or absence* of an MI, and in the *midsagittal area* of the MI are similar in terms of the process of sexual differentiation and/or functional significance.

#### Functional Significance

In contrast to the sexually dimorphic nuclei, many of which are believed to underlie sexually dimorphic reproductive functions, sexual dimorphism in the AC, MI, CC, and cerebral hemispheres are less dramatic and more difficult to explain. Whereas the sexually dimorphic nuclei are highly dimorphic as are the functions that they probably underlie, sex differences in regions not directly related to reproductive function are less dimorphic in terms of magnitude and exhibit considerable overlap among individuals of both genders in terms of the midsagittal area of the AC (figure 70.2a), MI (figure 70.2b), CC (Allen et al., '91), and cerebral hemispheres (Wada et al., '75). Although the functional significance of these structural sex differences is unknown, they are present in regions of the brain that control functions such as cerebral lateralization and cognitive skills that also exhibit only subtle sex differences with considerable overlap between male and female individuals. In fact, it is conceivable that differences in the AC and MI are of no functional significance but simply a result of different metabolic influences of estrogen (Curry and Heim, '66) and testosterone (Celotti et al., '87) on myelination. Should sex differences in the AC and/or MI reflect differences in the number of axons coursing through these structures, then these differences in "communication" may, in part, underlie sex differences in neurofunctional asymmetries (McGlone, '80; Beaton, '85; Kimura, '87) and/or cognitive function (Nyborg, '84; Kimura, '87). In fact, there is some indication that both the AC and MI may be involved in both cerebral lateralization and cognitive function.

**Anterior Commissure** The AC in macaques may play a role in inhibiting the bilateral formation of engrams, thereby increasing both functional asymmetry and pneumatic storage capacity of the brain by preventing redundancy (Doty and Overman, '77). The presence of the AC may be responsible for difficulties in discriminating left-right mirror images, since monkeys with sectioned ACs are able to differentiate more accurately between left-right mirror images (Achim and Corballis, '77). Together with the splenium of the CC, which may also be sexually dimorphic (de Lacoste-Utamsing and

Holloway, '82; Allen et al., '91), the AC mediates the interhemispheric transfer of visual discrimination between the hemispheres of nonhuman primates (Black and Myers, '64; Gazzaniga, '66; Noble, '68; Rocha-Miranda et al., '75; Gross et al., '77; Jouandet and Gazzaniga, '79) and in humans, the interhemispheric transfer of visual, auditory, and olfactory information (Risse et al., '78). However, it is unclear whether any of these specific functions is sexually dimorphic or whether the actual number of axons and/or midsagittal surface area of the AC has any bearing upon these functions.

**Massa Intermedia** In cats, the MI is involved in both the *symmetric* and *asymmetric* release of dopamine in the basal ganglia. With respect to the asymmetric effects, electrical stimulation of the forelimb (Leviel et al., '81) and certain dopaminergic drugs infused into the substantia nigra (Glowinski et al., '84) increase dopamine release from the ipsilateral caudate and decrease it from the contralateral caudate while decreasing it from the ipsilateral substantia nigra and increasing it from the contralateral substantia nigra. Similarly, unilateral infusions of GABA into the thalamic or intralaminar nuclei induce the release of dopamine in the contralateral substantia nigra (Romo et al., '84). The *anterior* part of the MI (interanteromedialis nucleus and nucleus reuniens), but not the nucleus centralis medialis, is involved in the bilateral regulation of dopamine from the nerve terminals and dendrites of the nigro-striatal dopaminergic neurons (Ch  ramy et al., '81). With respect to the *symmetric* effects upon dopamine release, unilateral electrical stimulation of the nucleus interanteromedialis, and nigral infusions of potassium (Glowinski et al., '84) result in increased *bilateral* release of dopamine from the caudate and substantia nigra (Glowinski et al., '84).

In the cat, the MI plays an important role both for transhemispheric ictal propagation and for the positive transfer effect of amygdaloid kindling (Hiyoshi and Wada, '88). In primates, there is the development of convulsive seizure bilateralization in animals with a sectioned hippocampal commissure, corpus callosum, and AC, probably due to communication between hemispheres through the MI (Wada and Mizoguchi, '84).

The role of the MI in asymmetries in the human brain is unknown; however, in the thalami, between which the MI is a major bridge, there are asymmetries of norepinephrine levels (Oke et al., '78). Furthermore, in humans, there is thalamic lateralization of language function (Riklan and Cooper, '77). Whether these asymmetries are due to axons that course through the MI or whether there are sex differences in thalamic nuclei is unknown; however, there are sex differences

in rats in striatal dopamine (Robinson et al., '80) and norepinephrine levels (Dark et al., '84). With respect to cognitive function, pneumoencephalograms demonstrated that men without an MI exhibited higher performance on the nonverbal portion of the Wechsler Bellevue IQ test than men with this structure (Lansdell and Davie, '72).

### Future Studies

Although it is unknown whether nonhuman animals have a sexually dimorphic AC or MI, animal models may be difficult to utilize for studying sexual dimorphism of the human AC and MI, since both structures change dramatically with advancing evolutionary development. For example, the AC in rodents consists predominantly of the anterior limb, which contains primarily axons from cell bodies of the olfactory system; however, in primates, the anterior limb may be reduced to a few strands and the posterior limb, which contains principally axons from cell bodies of the temporal cortex, is relatively evolved (Fox et al., '48; Klinger and Gloor, '60; Jouandet and Gazzaniga, '79). In contrast, the MI decreases in relative size as cortical evolution progresses (Sheps, '45; Glees and Wall, '48; Malobabic et al., '87). However, advancements in the resolution of in vivo imaging techniques such as magnetic resonance imaging may enable us to correlate the midsagittal areas of ACs and MIs with neuropsychological function and gonadal hormone exposure, thereby leading to an understanding of how environmental factors and/or the gonadal hormones sexually differentiate both the structure and function of the human brain. Since the AC, the MI, and possibly the splenium of the CC, are on the average larger at the midsagittal plane of the brain in human females, an interesting question arises: Is there greater connectivity between the cerebral hemispheres of women than in men?

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The possibility that neuroanatomical sex differences underlie functional sex differences has been studied extensively in nonhuman animals. In regions of the CNS known to control sex-related behaviors, sexual dimorphisms range from subtle differences in the size of individual neurons (Pfaff, 1966), dendritic branching patterns (Greenough et al., 1977), and synaptic organization (Raisman and Field, 1971), to dramatic dimorphisms in the volumes of cell groups (Nottebohm and Arnold, 1976; Gorski et al., 1978). In addition, several sexual dimorphisms have been found in regions not necessarily involved in reproductive function. For example, in rats, there are sexually dimorphic patterns of cortical and hippocampal asymmetries (Diamond et al., 1983). While several neuroanatomical sexual dimorphisms are influenced by perinatal environmental factors, nearly all identified sex differences are known to be influenced by gonadal hormones perinatally and/or during adulthood.

In contrast to what is known in laboratory animals, there are relatively few reported sex differences in the human CNS. In regions possibly influencing reproductive function, there are several sex differences: relatively marked sexual dimorphisms exist in cell groups in the preoptic-anterior hypothalamic area (Swaab and Fliers, 1985; Allen et al., 1989), a region that has been implicated in rodents and subhuman primates in gonadotropin release (Gorski, 1968; Plant et al., 1979), maternal behavior (Jacobson et al., 1980), and sexual behavior (Robinson and Mishkin, 1966); similarly, a region of the bed nucleus of the stria terminalis is larger in males than in females (Allen and Gorski, 1990), the shape of the suprachiasmatic nucleus differs between men and women (Swaab et al., 1985), and Onuf's nucleus in the spinal cord, which innervates perineal muscles, contains more motoneurons in the male than in the female (Forger and Breedlove, 1986).

Recent reports suggest that sex differences in non-reproductive functions in humans may also have a neuroanatomical basis. Such functional sex differences may include the following: (1) cognitive decline with advancing age (Tomlinson and Corsellis, 1984); (2) subtle differences in cognitive skills whereby women

generally perform better on verbal measures and men perform better in spatial and mathematical skills (Harris, 1978; Kimura, 1987); (3) a prevalence in boys relative to girls of a variety of developmental language disorders including delayed speech acquisition, dyslexia, infantile autism, and stuttering (Hier, 1979); and (4) greater functional asymmetry in the male than in the female brain (McGlone, 1980; Beaton, 1985; Kimura, 1987). Several structural sexual dimorphisms suggest that there may be a morphological basis for these functional differences. Moreover, possible gender differences in cognitive decline with age could correspond with age-related sex differences in decreases in brain size (Hatazawa et al., 1982; Hubbard and Anderson, 1983). In men, just as there appears to be greater functional asymmetry, there is greater morphological brain asymmetry in the temporal planum (Wada et al., 1975). In women, there may be greater connectivity between the 2 hemispheres of the brain: the massa intermedia of the thalamus is more often present (Rabl, 1958), and both the massa intermedia (Allen and Gorski, 1987) and the anterior commissure (Allen and Gorski, 1986) are larger at the midsagittal plane in women than in men. Similarly, sex differences in the corpus callosum (CC) were originally reported by de Lacoste-Utamsing and Holloway (1982), but have subsequently become controversial. This is the first study that replicates each of the 4 original measurements of de Lacoste-Utamsing and Holloway (1982), uses age-matched male and female subjects, and contains the largest sample size to date. In addition, the CC has been examined extensively for sex differences in its shape and the area of many regional components.

#### Materials and Methods

We examined the magnetic resonance images (MRIs) of the midsagittal plane of each human brain that was scanned at 4 southern California MRI centers before locating a center that had enough midsagittal images from subjects who had "normal" scans according to the radiologists' reports to obtain a minimum of 100 age-matched male and female subjects. All images in

this study were taken at the University of California at Irvine Medical Center using a headcoil of a Technicare Teslacon 2.0-tesla superconductive magnet operating at 0.6 tesla. Each single-echo midsagittal acquisition was 0.75 cm thick and obtained for T1 or T2 weighting.

From approximately 1000 sets of MRIs of the brains of human beings, each set of which contained a midsagittal section, 246 midsagittal images were selected, without knowledge of age or gender, on the basis of the following: (1) precision of the midsagittal plane determined by the callosal sulcus separating the CC from the cingulate gyrus, the appearance of the cerebral aqueduct between the tectum and tegmentum, the V-shaped roof of the fourth ventricle, the presence of the cerebellar vermis, and the complete absence of the cerebellar hemispheres; (2) quality of image such as freedom from any head motion during imaging; and (3) general absence of neuropathology determined by a "normal" MRI according to the radiologist's report and no history of neuroendocrine abnormality, neurosurgery, or previous diagnosis of a neurological disorder known to affect gross neural structure. Although most subjects, except for several controls, were scanned for a medical reason, a majority of which included sinusitis, headache, dizziness, sensory problems, fainting, and nausea, there is no reason to believe that these problems would influence neural structure detectable by an MRI that appeared "normal." Subsequently, without reference to the image itself, the MRI codes of these 246 individuals, with their sex and age, were separated into 2 columns based on gender and ordered according to age. Since not all subjects were paired, due to age and sex discrepancies, this resulted in 73 age-matched pairs with no more than 5 yr between any pair. Using age 16 as the beginning of adulthood, these subjects were separated into 12 pairs of children and 61 pairs of adults.

The midsagittal images from each of the 146 individuals, as well as a standard grid used for evaluating magnification, were photographed and made into slides (figure 71.1A,B). The images were projected at a magnification of either 1.225 $\times$  or 4 $\times$  onto a flat projection surface covered by white drawing paper. To avoid distortion of the midsagittal surface of the brain, correct alignment of the slide projector with the projection surface was achieved by measuring the squares of the grid in different regions on the projection surface and making minor adjustments in the position of the projector, until the sides of the squares of the grid were of equal length when projected.

Without knowledge of age or sex, 2 individuals first traced each image of the midsagittal surface of the brain, which included the cerebral cortex, CC, cerebellum, midbrain, pons, and medulla, at a magnification of 1.225 $\times$ . Because the curved and convoluted outer

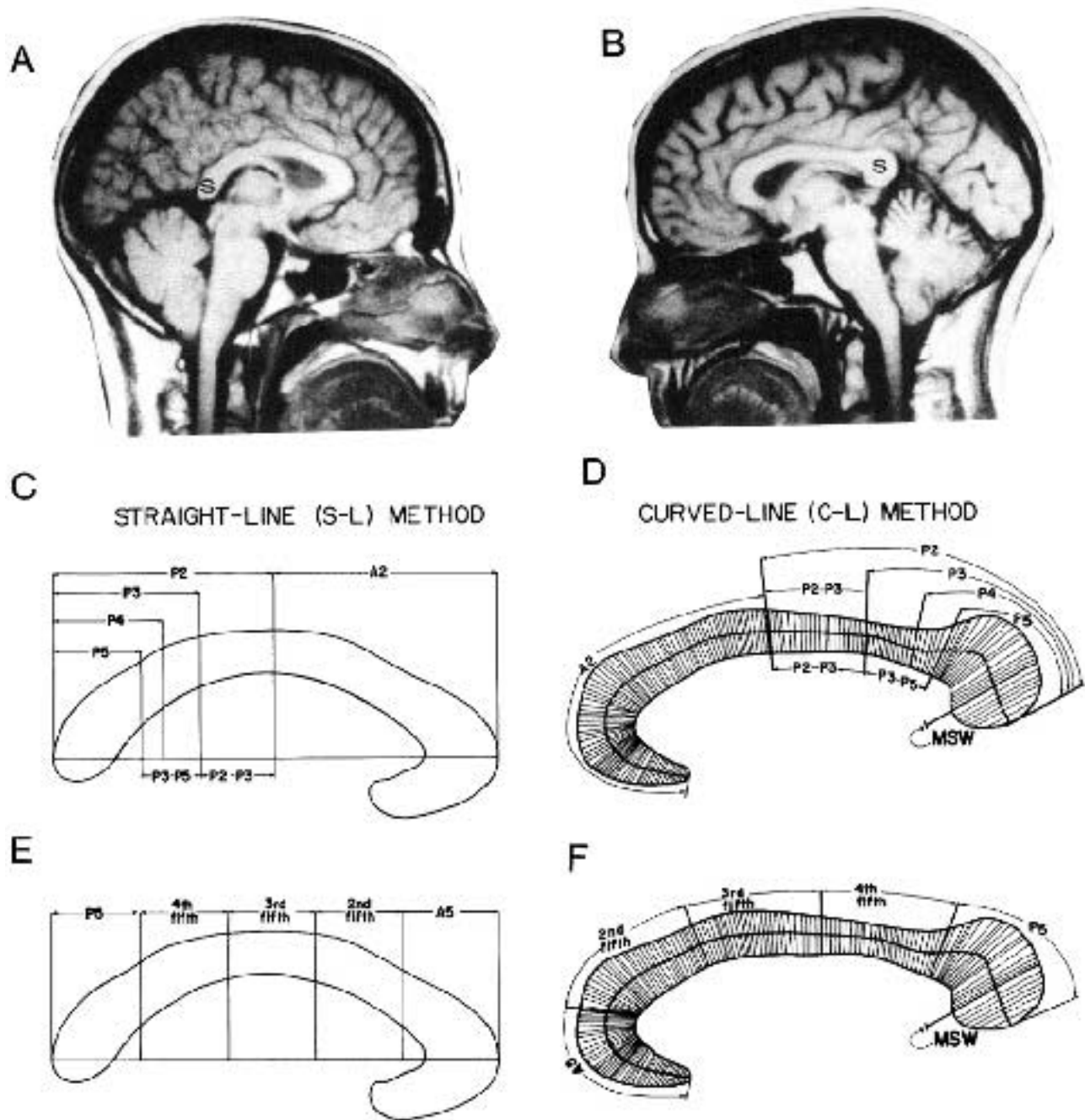
surface of the cerebral cortex frequently did not have clearly defined borders, its boundary was determined by the relatively distinct dura mater. Subsequently, the projector was realigned at a magnification of 4 $\times$ , and the midsagittal outline of the CC was drawn.

Each investigator made 2 photocopies of the drawings of the CC. The first set of drawings was evaluated by dividing the CC using the "straight-line method" (S-L method; figure 71.1C,E), which took into account the distance from the most rostral to the most caudal point of the CC along a straight line. This straight line was used to divide the CC into fifths [from anterior to posterior: anterior  $\frac{1}{5}$  (A5), 2nd  $\frac{1}{5}$ , 3rd  $\frac{1}{5}$ , 4th  $\frac{1}{5}$ , and posterior  $\frac{1}{5}$  (P5)], anterior half (A2), posterior half (P2), posterior third (P3), and posterior fourth (P4), by drawing lines perpendicular to the straight line. The second set of drawings was analyzed by the "curved-line method" (C-L method; figure 71.1D,F), which measured the length of the curved line bisecting the ventral and dorsal halves of the CC from the rostrum to the splenium. This curved line was obtained by drawing adjacent lines, at intervals of no more than 2 mm apart, as perpendicularly as possible between the ventral and dorsal surface of the CC, determining the midpoint of each line, and connecting these midpoints to obtain the curved, bisecting line. The length of this curved line was measured using the Bioquant Hipad digitizer (Bioquant IBM version 2.1, R & R Biometrics), which had a resolution of 0.5 mm. Similarly, this curved line was divided into each  $\frac{1}{5}$ , A2, P2, P3, and P4 by drawing lines perpendicular to the tangent of the bisecting line. From these measures, the areas within P2 minus P3 (P2 - P3) and P3 - P5 were calculated using both the S-L and C-L methods of partitioning. P2 - P3 and P3 - P5 were quantified because they may contain axons from asymmetric regions of the cerebral cortex and have been found to differ between men and women in relation to hand preference (Witelson, 1986, 1989).

We evaluated the shape of the posterior CC for possible sex differences, utilizing 3 criteria: (1) subjective classification of gender based on the shape of the posterior CC, (2) maximum splenium width (MSW), and (3) bulbosity coefficients.

(1) To determine whether the CC could be evaluated subjectively according to gender, based on reports of a more bulbous-shaped female splenium and a more tubular-shaped male splenium, 3 investigators, without knowledge of the subjects' gender, divided the CC drawings into male and female groups. The subjects' gender was based on the classification by a minimum of 2 of the investigators.

(2) Because the CC is a continuous structure without defined internal dimensions, and because the splenium is nonspecifically defined as the "thickened posterior

**Figure 71.1**

Sexual dimorphism in the splenium of the CC. *A* and *B* are MRIs of the mid-sagittal section of the human CC from a male (*A*) and a female (*B*), illustrating a more tubular-shaped male splenium (*S*) and a more bulbous-shaped female splenium (*S*), respectively. *C* and *E*, tracings of the CC from *A*, illustrate the S-L method of partitioning. *D* and *F*, tracings of the CC from *B*, illustrate the C-L method of partitioning and the method of obtaining the MSW.

**Table 71.1**Mean  $\pm$  SEM for measurements of the cerebral cortex, CC, MSW, minimum body width, and bulbosity coefficients

	All Subjects			Adults			Children		
	Males	Females	<i>p</i>	Men	Women	<i>p</i>	Boys	Girls	<i>p</i>
Cerebral cortex (cm <sup>2</sup> )	93.64 $\pm$ 0.73	89.93 $\pm$ 0.65	0.002*	92.96 $\pm$ 0.77	88.57 $\pm$ 0.69	0.006*	97.09 $\pm$ 1.95	92.98 $\pm$ 1.67	0.2
CC (cm <sup>2</sup> )	6.81 $\pm$ 0.09	6.63 $\pm$ 0.07	0.26	6.87 $\pm$ 0.10	6.80 $\pm$ 0.07	0.68	6.49 $\pm$ 0.26	5.76 $\pm$ 0.18	0.1
MSW (cm)	1.25 $\pm$ 0.02	1.30 $\pm$ 0.02	0.12	1.27 $\pm$ 0.02	1.35 $\pm$ 0.02	0.035*	1.14 $\pm$ 0.035	1.07 $\pm$ 0.04	0.16
Minimum body width (cm)	0.52 $\pm$ 0.01	0.50 $\pm$ 0.01	0.15	0.53 $\pm$ 0.01	0.52 $\pm$ 0.01	0.63	0.51 $\pm$ 0.02	0.42 $\pm$ 0.02	0.01*
Bulbosity coefficients									
Splenium relative to 4th $\frac{1}{5}$ (%)	61.0 $\pm$ 2.9	72.4 $\pm$ 3.6	0.015*	62.7 $\pm$ 3.1	73.2 $\pm$ 3.8	0.038*	52.5 $\pm$ 8.0	68.5 $\pm$ 9.9	0.23
Splenium relative to P2 (%)	62.4 $\pm$ 2.7	76.9 $\pm$ 4.0	0.002*	64.3 $\pm$ 2.9	78.6 $\pm$ 4.5	0.006*	52.9 $\pm$ 6.3	68.5 $\pm$ 8.0	0.17
Splenium relative to CC (%)	49.7 $\pm$ 2.5	56.9 $\pm$ 2.5	0.033*	52.6 $\pm$ 2.7	58.9 $\pm$ 2.7	0.09	35.3 $\pm$ 4.7	46.8 $\pm$ 5.9	0.16

Data are presented as mean  $\pm$  SEM, *p*, probability using the paired *t* test.

extremity of the CC,” we considered P5 of the CC to be the splenium (de Lacoste-Utamsing and Holloway, 1982). The MSW was determined by finding the maximum distance perpendicular to the tangent of the bisecting line in the P5 of the CC (figure 71.1E). Similarly, the minimum width of the body of the CC was determined by finding the minimum distance perpendicular to the tangent of the bisecting line anterior to P5 and posterior to the rostrum.

(3) To quantify the bulbosity of P5, we utilized a “bulbosity coefficient.” This is a measure that compares the average width of the splenium with the average width of an adjacent region of the CC. Specifically, the bulbosity coefficient is the percentage by which the average width of P5 is wider, or more “bulbous,” than the average width of the adjacent CC. To derive the bulbosity coefficient, we first obtained the average width of a region by dividing its area by the length of the curved line coursing through it. For example, the average width of P5 is the area of P5 divided by the length of the C-L through P5. Second, we subtracted the average width of the adjacent region from the average width of P5 and divided by the width of the adjacent region. This number was multiplied by 100 to obtain the percentage. For example, the bulbosity coefficient for P5 in relation to P2 is calculated as follows:

$$[(\text{average width of P5} - \text{average width of P2 not including P5}) / (\text{average width of P2 not including P5})] \times 100$$

We obtained bulbosity coefficients for P5 in relation to the adjacent  $\frac{1}{5}$  (4th  $\frac{1}{5}$ ), P2, and the CC.

Both investigators quantified the area of the cerebral cortex, CC, and the components of the CC in both their S-L- and their C-L-partitioned set of drawings, using the Bioquant Hipad digitizer. In addition, each investigator measured the MSW, minimum width of the body, and both the straight length and the curved length of the CC. Each measurement of the 2 investiga-

tors was compared, and if there was a difference of greater than 5% between measurements, the appropriate region(s) of the CC was reexamined by a third person and the 2 measurements in closest agreement were utilized. Each of these pairs of measurements was averaged.

For both children and adults, each measurement was examined for changes with advancing age using Pearson’s correlation coefficient (Dawson-Saunders and Trapp, 1990). Each measure was further examined for girls, boys, women, and men separately, and stepwise regression was used to determine whether the regression slopes differed between girls and boys and between women and men. The cerebral cortex was of particular interest in this respect because we had originally planned to account for sex differences in brain size by adjusting our measurements of the CC to the area of the cerebral cortex, though this was not possible because of a difference in regression slopes between women and men (see Results). Measurements of the CC were examined using the paired *t* test, and subjective classification of gender based on the shape of the posterior CC was examined with  $\chi^2$ . Because only 1 of the 23 area measurements of the CC and its subdivisions exhibited a significant sex difference, which would be expected by chance, we evaluated it (P2 – P3) with the Bonferroni *t* method for multiple comparisons (Dunn’s multiple-comparison procedure; Dawson-Saunders and Trapp, 1990).

## Results

The present results are based on an analysis of the brains of 73 age-matched pairs of males and females (tables 71.1–71.3). Among the children, the age range in years for girls was 2 to 15 (Mean  $\pm$  SEM = 9.25  $\pm$  3.8) and for boys was 2 to 14 (8.9  $\pm$  3.8), with an average absolute difference between pairs of 1 yr. Among adult subjects, the age range in years for men

**Table 71.2**Mean  $\pm$  SEM for measurements of the CC with the S-L and C-L methods

	All Subjects			Adults			Children		
	Males	Females	<i>p</i>	Men	Women	<i>p</i>	Boys	Girls	<i>p</i>
<i>S-L method</i>									
Length (cm)	7.18 $\pm$ 0.04	7.14 $\pm$ 0.04	0.63	7.24 $\pm$ 0.04	7.18 $\pm$ 0.04	0.46	6.86 $\pm$ 0.09	6.96 $\pm$ 0.08	0.50
P5 (cm <sup>2</sup> )	1.88 $\pm$ 0.03	1.90 $\pm$ 0.03	0.77	1.92 $\pm$ 0.03	1.96 $\pm$ 0.03	0.55	1.67 $\pm$ 0.08	1.58 $\pm$ 0.06	0.46
P4 (cm <sup>2</sup> )	2.17 $\pm$ 0.03	2.18 $\pm$ 0.03	0.91	2.22 $\pm$ 0.03	2.25 $\pm$ 0.03	0.65	1.91 $\pm$ 0.09	1.81 $\pm$ 0.07	0.81
P3 (cm <sup>2</sup> )	2.55 $\pm$ 0.04	2.54 $\pm$ 0.04	0.83	2.61 $\pm$ 0.04	2.62 $\pm$ 0.04	0.83	2.29 $\pm$ 0.10	2.12 $\pm$ 0.09	0.28
P2 (cm <sup>2</sup> )	3.30 $\pm$ 0.04	3.24 $\pm$ 0.04	0.42	3.36 $\pm$ 0.05	3.34 $\pm$ 0.04	0.80	3.01 $\pm$ 0.13	2.73 $\pm$ 0.11	0.16
A2 (cm <sup>2</sup> )	3.43 $\pm$ 0.05	3.35 $\pm$ 0.04	0.35	3.42 $\pm$ 0.05	3.42 $\pm$ 0.04	0.98	3.49 $\pm$ 0.13	3.00 $\pm$ 0.09	0.05*
P2 – P3 (cm <sup>2</sup> )	0.75 $\pm$ 0.02	0.70 $\pm$ 0.02	0.06	0.75 $\pm$ 0.02	0.72 $\pm$ 0.02	0.19	0.72 $\pm$ 0.05	0.61 $\pm$ 0.04	0.08
P3 – P5 (cm <sup>2</sup> )	0.67 $\pm$ 0.01	0.64 $\pm$ 0.01	0.19	0.68 $\pm$ 0.02	0.66 $\pm$ 0.01	0.46	0.62 $\pm$ 0.03	0.54 $\pm$ 0.03	0.11
<i>C-L method</i>									
Length (cm)	9.10 $\pm$ 0.06	9.04 $\pm$ 0.05	0.57	9.16 $\pm$ 0.07	9.09 $\pm$ 0.05	0.57	8.78 $\pm$ 0.11	8.78 $\pm$ 0.11	1.00
P5 (cm <sup>2</sup> )	1.84 $\pm$ 0.03	1.86 $\pm$ 0.03	0.63	1.88 $\pm$ 0.03	1.92 $\pm$ 0.03	0.40	1.64 $\pm$ 0.08	1.54 $\pm$ 0.07	0.38
P4 (cm <sup>2</sup> )	2.17 $\pm$ 0.03	2.18 $\pm$ 0.03	0.85	2.22 $\pm$ 0.04	2.25 $\pm$ 0.03	0.57	1.93 $\pm$ 0.08	1.81 $\pm$ 0.08	0.33
P3 (cm <sup>2</sup> )	2.62 $\pm$ 0.04	2.61 $\pm$ 0.04	0.86	2.67 $\pm$ 0.04	2.69 $\pm$ 0.04	0.78	2.37 $\pm$ 0.10	2.19 $\pm$ 0.09	0.25
P2 (cm <sup>2</sup> )	3.54 $\pm$ 0.05	3.46 $\pm$ 0.05	0.32	3.60 $\pm$ 0.05	3.57 $\pm$ 0.04	0.72	3.26 $\pm$ 0.14	2.93 $\pm$ 0.11	0.12
A2 (cm <sup>2</sup> )	3.19 $\pm$ 0.04	3.12 $\pm$ 0.04	0.41	3.19 $\pm$ 0.05	3.19 $\pm$ 0.04	0.96	3.20 $\pm$ 0.12	2.79 $\pm$ 0.08	0.09
P2 – P3 (cm <sup>2</sup> )	0.92 $\pm$ 0.02	0.85 $\pm$ 0.02	0.01**	0.93 $\pm$ 0.02	0.88 $\pm$ 0.02	0.08	0.90 $\pm$ 0.06	0.74 $\pm$ 0.04	0.04**
P3 – P5 (cm <sup>2</sup> )	0.78 $\pm$ 0.01	0.75 $\pm$ 0.01	0.12	0.80 $\pm$ 0.01	0.77 $\pm$ 0.01	0.30	0.73 $\pm$ 0.04	0.65 $\pm$ 0.03	0.18

Data are presented as mean  $\pm$  SEM, *p*, probability using the paired *t* test. For simplicity, the means for A5, 2nd  $\frac{1}{5}$ , 3rd  $\frac{1}{5}$ , and 4th  $\frac{1}{5}$  are not given; however, none exhibited sexual dimorphism. \*, *p* = 0.052, NS.

**Table 71.3**

Correlation coefficients for changes with advancing age

	Children		Adults	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Area of cerebral cortex	0.074	0.73	0.06	0.472
Area of CC	0.601	0.0019*	–0.19	0.032*
MSW	0.639	0.0008*	0.012	0.897
Minimum body width	0.504	0.012*	–0.037	0.682
Straight length	0.690	0.0002*	0.059	0.514
Curved length	0.580	0.003*	–0.025	0.784
Areas within the CC				
A5	0.454	0.026*	–0.218	0.016*
2nd $\frac{1}{5}$	0.492	0.015*	–0.300	0.0008*
3rd $\frac{1}{5}$	0.503	0.012*	–0.234	0.009*
4th $\frac{1}{5}$	0.444	0.029*	–0.068	0.458
A2	0.498	0.013*	–0.298	0.0008*
P2	0.645	0.0007*	–0.067	0.462
P3	0.659	0.0005*	–0.046	0.612
P4	0.682	0.003*	–0.055	0.546
P5	0.712	0.0001*	–0.034	0.710

This table shows Pearson's correlation coefficients (*r*) and probabilities (*p*) for changes with advancing age. These values only represent areas of the CC that have been partitioned using the C-L method (see Figure 71.1D); however, the corresponding S-L-partitioned areas exhibited similar values.

was 16 to 78 ( $42.1 \pm 14.0$ ) and for women was 16 to 79 ( $41.9 \pm 13.6$ ), with an average difference between pairs of 1.2 yr.

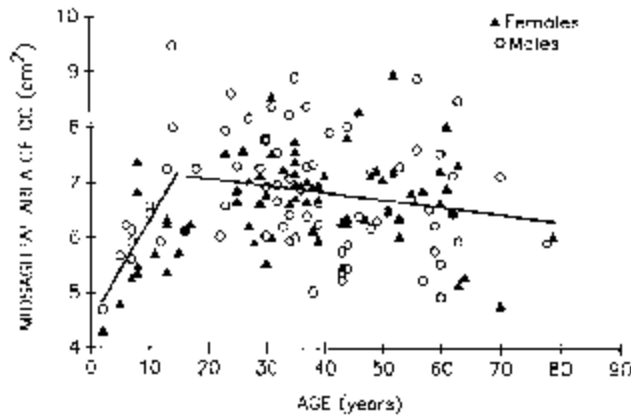
#### Correlation between Measurements

Throughout this study, there was a highly significant correlation between each measurement of the 2 experimenters ( $P < 0.0001$ ).

Whether we examined males and females separately or as a group, when comparing the area of the various subdivisions of the CC utilizing the S-L and the C-L method, there was a significant difference between each measurement except for P4. The regions A2, A5, and P5 were significantly larger in area when defined by the S-L method than by the C-L method, whereas the 2nd, 3rd, and 4th  $\frac{1}{5}$ , as well as P2, P3, P2 – P3, and P3 – P5, were greater when defined by the C-L method than by the S-L method. However, no trend was observed for either method to increase the probabilities of significant sex differences (table 71.2).

#### Changes with Advancing Age

We examined children and adults separately for age-related changes in the cerebral cortex, the entire CC, and 22 components of it. Among the children, the CC and each of its regions *increased* significantly with advancing age (table 71.3); however, the increase was not significant in the midsagittal area of the cerebral cortex. When regression slopes were compared between girls and boys, A5 ( $p < 0.05$ ), 2nd  $\frac{1}{5}$  ( $p < 0.05$ ), 3rd  $\frac{1}{5}$  ( $p < 0.02$ ), and A2 ( $p < 0.02$ ), utilizing both the S-L



**Figure 71.2**

Midsagittal area of the CC in relation to age. The area of the CC and age are correlated for both children (2–15 yr of age;  $r = 0.60$ ;  $p = 0.0019$ ) and for adults (16–79 yr of age;  $r = 0.19$ ;  $p = 0.032$ ). There was no significant difference in regression slopes between boys and girls or between men and women. The ascending and descending lines represent the regression slopes for children and adults, respectively.

and the C-L methods of partitioning, exhibited significantly greater increases with advancing age in boys than in girls. In adults, the areas of the CC, A5, 2nd and 3rd  $\frac{1}{5}$ , and A2, *decreased* significantly with advancing age. The only measure that exhibited significantly different regression slopes between women and men was the midsagittal area of the cerebral cortex that, though decreasing insignificantly in adults as a group ( $r = 0.065$ ;  $p = 0.477$ ), decreased significantly in women ( $r = -0.268$ ;  $p = 0.037$ ) but not in men ( $r = 0.1038$ ;  $p = 0.426$ ).

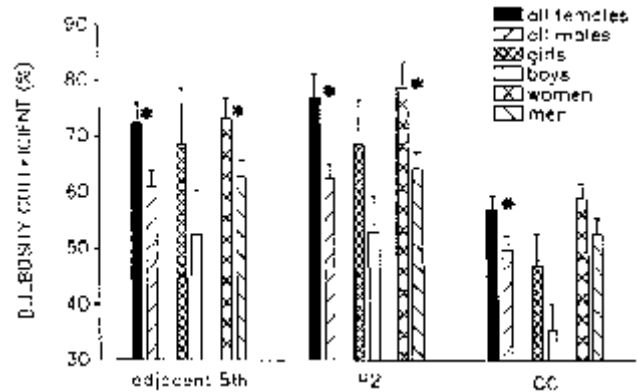
#### Size of the CC

Although the overall area of the CC was slightly greater in men than in women (1.0%;  $p = 0.68$ ) and in boys than in girls (12.5%;  $p = 0.102$ ; table 71.1, figure 71.2), this difference was considerably less than the reported sex differences in brain weight (de Lacoste-Utamsing and Holloway, 1982). The CC ranged from 4.31 to 8.97 cm<sup>2</sup> for females and from 4.70 to 9.474 cm<sup>2</sup> for males; hence, there was considerable overlap in the size of the CC between the male and female subjects.

#### Sex Differences in the Shape of the CC

There were striking sex differences in the shape of the splenium, utilizing 3 criteria for evaluation: (1) subjective classification of the gender based on the shape of the posterior CC, (2) MSW, and (3) bulbosity coefficients.

1. Subjective classification of the posterior CC of all subjects by sex based on a more bulbous-shaped female splenium and a more tubular-shaped male splenium



**Figure 71.3**

The bulbosity coefficients, which represent the percentage by which the splenium (P5) of the CC is wider, or more “bulbous,” than the adjacent regions of the CC, are compared between females and males, between girls and boys, and between women and men. The adjacent fifth compares the splenium with the 4th  $\frac{1}{5}$  of the CC, P2 compares it to the remaining posterior half, and CC compares it to the remaining CC. Asterisks denote significant differences ( $p < 0.05$ ; see table 71.3).

revealed a significant correlation between the observers’ sex rating based on shape and the actual gender of the subject ( $\chi^2 = 13.2603$ ; 1 df; contingency coefficient = 0.289;  $p < 0.0003$ ). Specifically, 80 out of 122 (66%) of the adults’ CC ( $\chi^2 = 10.623$ ; 1 df; contingency coefficient = 0.283;  $p < 0.0011$ ) and 16 out of 24 (67%) of the childrens’ CC ( $\chi^2 = 1.500$ ; 1 df; contingency coefficient = 0.243) were correctly identified, however, this is not more accurate than could be achieved by chance ( $p < 0.2207$ ).

2. The degree of bulbosity or tubularness of the splenium was ascertained as in the original study (de Lacoste-Utamsing and Holloway, 1982) by measuring the MSW (table 71.1). We found that the MSW was significantly greater in women than in men both before ( $p = 0.035$ ) and after ( $p = 0.01$ ) the area of the CC was considered. However, in children, there was no significant sex difference in MSW; though its absolute value was greater in boys than in girls, its value adjusted for CC area was greater in girls.

3. The bulbosity coefficients, which represent the percentage by which the average width of the splenium is greater than the average width of adjacent regions of the CC, were all significantly greater in females than in males (table 71.1, figure 71.3). Specifically, the bulbosity coefficient was 18.7% greater in females in relation to the adjacent  $\frac{1}{5}$  ( $p = 0.015$ ), 23.2% greater in relation to the remaining posterior half ( $p = 0.002$ ), and 14.5% greater in relation to the remaining CC ( $p = 0.032$ ). These percentage differences were even greater for children: the bulbosity coefficient was 30.5% greater in girls in relation to the adjacent  $\frac{1}{5}$ , 29.5% greater in relation to the remaining posterior half, and

32.6% greater in relation to the remaining CC. In contrast to males and females as a group, however, these values for children were not significant in the 2 sexes, perhaps because of the small sample size ( $N = 12$  pairs).

#### Regional Sex Differences in the CC

Regions of the CC were examined using both the S-L and the C-L methods of partitioning in terms of each  $\frac{1}{5}$ , P4, P3, P2, A2, P2 – P3, P3 – P5, the minimum body width, S-L length, and C-L length (figure 71.1, table 71.2). The only region that exhibited sexual dimorphism was P2 – P3 examined by the C-L method, which was greater in males ( $p = 0.01$ ) and boys ( $p = 0.04$ ). However, because 23 measurements of area were compared, including the CC and 22 subdivisions, at least 1 significant difference would be expected by chance at the level of  $p < 0.05$ ; therefore, we examined P2 – P3 utilizing the conservative Bonferroni adjustment, in which case P2 – P3 was not sexually dimorphic statistically. In addition, each of these regions was adjusted for CC area by dividing by the respective CC area (calculations not shown). Following this adjustment, the only significant sex difference observed was a greater P5 in females ( $p = 0.033$ ) and a greater P2 – P3 in males ( $p = 0.021$ ) when examined by the C-L method, however, these differences were no longer present following the Bonferroni adjustment. Interestingly, the cross-sectional area of the splenium (P5) and its value adjusted for CC area was insignificantly greater in women than in men, using both methods of measurement. In children, though the cross-sectional area of the splenium was greater in boys than in girls, its area adjusted for CC area was greater in girls than in boys, again using both the S-L and the C-L methods. Note, however, that none of these differences were statistically significant in children alone, even without the Bonferroni  $t$  method of adjustment for multiple comparisons.

#### Correlations between the CC and its Components

There was a significant correlation between the area of the CC and each of its area subdivisions ( $r > 0.7$ ;  $p < 0.001$ ), in addition to each linear measure (the curved length, straight length, minimum distance, and MSW,  $r > 0.42$ ;  $p < 0.02$ ) for all subjects, adults, men, women, children, boys, and girls.

#### Discussion

##### Methodological Considerations

It is unclear whether the S-L or the C-L method of analysis is more appropriate for consistently partitioning the CC into regions that are more similar in terms of topographical organization of projections from dif-

ferent cortical areas. However, the C-L method is necessary for evaluating the CC in terms of sex differences in shape. The CC was occasionally difficult to trace at the tip of the rostrum, and this may add to error in the C-L partitions, while affecting only the rostral S-L division. Although there were significant differences between the C-L and the S-L measurements in each region except for P4, these differences were in the same direction for both genders.

##### Changes with Advancing Age

In children, all examined regions of the CC increased significantly with advancing age (table 71.3). Furthermore, the anterior regions of the CC increased to a significantly greater extent in boys than in girls; however, it is unclear whether the CC in boys actually grows more rapidly than in girls, or whether the significant difference between regression slopes for area changes with advancing age reflects the fact that the largest of the 146 CC was of the oldest boy.

In adults, the anterior regions of the CC (A2, A5, 2nd  $\frac{1}{5}$ , and 3rd  $\frac{1}{5}$ ), which carry axons from the frontal, premotor, and motor cortices, decrease in area significantly with advancing age; however, these changes are not significant in the posterior regions of the CC, which contain fibers from the somesthetic, parietal, occipital, and temporal lobes. It is unclear why the midsagittal area of the cerebral cortex was the only parameter of those measured that exhibited significant differences in regression slopes between women and men. However, several studies report that a decrease in brain volume occurs earlier in women than in men (Hatazawa et al., 1982; Hubbard and Anderson, 1983). Furthermore, in Alzheimer's disease, there is both improvement of cognitive function in some women who are treated with estrogen (Fillit et al., 1986) and a significant loss of large neurons in the midfrontal cortex in women but not men (Terry et al., 1981).

##### Sex Differences in the Anatomy of the CC

Although the CC is the largest interhemispheric commissure, composed of about  $200\text{--}350 \times 10^6$  fibers connecting the right and left cerebral hemispheres, the axons that constitute it come from only approximately 2% of all neocortical neurons (Berlucchi, 1981). Investigations in both humans and other primates suggest that most callosal fibers arise from the association cortex and project to a homotopic position on the contralateral hemisphere. For example, in the human, both the superior parietal lobule and the occipital cortex give rise to fibers that course through the splenium (de Lacoste et al., 1985) which appear to serve in the interhemispheric transfer of visual information (Gordon et al., 1971; Gazzaniga and Freedman, 1973). Of similar functional interest is the posterior body of the

CC (approximately P3 – P5 and P2 – P3; Witelson, 1989) which carries fibers connecting the posterior parietal and temporal regions of the 2 hemispheres involved in language function (monkey, Seltzer and Pandya, 1983; Cipolloni and Pandya, 1985; man, de Lacoste et al., 1985).

#### Discrepancies in the Literature

Although several investigators have attempted to replicate the original report (de Lacoste-Utamsing and Holloway, 1982) of sex differences in the CC, no other subsequent study has actually evaluated the same 4 measurements, which include (1) the area of the CC, (2) the MSW, (3) the area of P5, and (4) subjective gender classification based on the shape of the splenium. de Lacoste-Utamsing and Holloway (1982) observed that the MSW was significantly greater in females, and each investigator correctly identified the gender of the CC of each subject, based on the shape of the CC. However, they did not describe their method of evaluating the shape of the splenium until a subsequent study (de Lacoste et al., 1986). Thus, only a few investigators made similar measurements (Bell and Variend, 1985; Holloway and de Lacoste, 1986; Demeter et al., 1988; Witelson, 1989) while others made different measurements (Weber and Weis, 1986; Oppenheim et al., 1987; Byne et al., 1988; Weis et al., 1988; Clarke et al., 1989). We observed that, though gender in our sample could not always be accurately determined from the CC, there was a significant association between the shape of the splenium and the gender of the subject. Furthermore, among our 27 absolute measures of the CC, only the MSW and P2 – P3 (C-L method; utilizing paired *t* test, but not Bonferroni adjustment for multiple comparisons) were sexually dimorphic. While other studies report that gender cannot be absolutely determined by the shape of the posterior CC (Weber and Weis, 1986; Byne et al., 1988; Weis et al., 1988), each study reporting subjective gender classification based on the shape of the splenium (Bell and Variend, 1985; Weber and Weis, 1986; Yoshii et al., 1986; Kertesz et al., 1987; Oppenheim et al., 1987) was able to identify correctly the gender in more than half of their subjects.

While some investigators have determined the regional areas of the CC by dividing it in a manner similar to the S-L method (Bell and Variend, 1985; Witelson, 1985; Weber and Weis, 1986; Oppenheim et al., 1987; Byne et al., 1988; Demeter et al., 1988; Clarke et al., 1989; Witelson et al., 1989), others have divided the CC in a manner similar to the C-L method (Nasrallah et al., 1986; Demeter et al., 1988; Clarke et al., 1989). However, it is important for investigators to measure the CC in a similar manner in order to compare results. In our data, P5, P4, and P3 adjusted

for CC area are slightly larger in area in women than in men. Other investigators also have observed that the posterior components of the CC are slightly larger in females (de Lacoste-Utamsing and Holloway, 1982; Oppenheim et al., 1987; Byne et al., 1988; Weis et al., 1988), particularly when adjusted for CC area (Demeter et al., 1988; Clarke et al., 1989;  $p < 0.05$ ). While several investigations report that the area of the CC is greater in females (de Lacoste-Utamsing and Holloway, 1982; de Lacoste et al., 1986; Holloway and de Lacoste, 1986; Byne et al., 1988), most studies report that it is greater in males (Nasrallah et al., 1986; Weber and Weis, 1986; Kertesz et al., 1987; Demeter et al., 1988; Weis et al., 1988); however, it tends to be greater in females when adjusted for sex differences in brain weight (de Lacoste-Utamsing and Holloway, 1982; Holloway and de Lacoste, 1986).

Although several studies report no significant decrease in CC area with aging, in our relatively larger group of adults, we did observe such a decrease with advancing age. Similarly, regions of the CC decreased significantly with age in both genders. Because neural atrophy does occur with aging, both the failure to age-match subjects and differences in age distribution may contribute to these conflicting results. We know of no other study that specifically age-matched its subjects; in fact, several did not mention the age of subjects (de Lacoste-Utamsing and Holloway, 1982; Oppenheim et al., 1987). In several studies, sex differences in the area of the CC appear to correspond to sex differences in the age of the male and female groups. Specifically, in an autopsy sample where the men were 19 yr younger than the women (Clarke et al., 1989;  $p < 0.0001$ ), the CC of men were 15% larger than those of females. Likewise, in a group where females were an average of 10 yr younger than the males, the CC of females were 16% larger than those of males (Byne et al., 1988). The sex difference in the area of the CC appears to decrease when males and females are of similar ages. In a sample where the females were only 9 months younger than males, the CC of females were only 2% larger than those of males (Clarke et al., 1989). Similarly, in this study where the females were only 2.4 months younger than the males, the CC were only 1% larger in males. Thus, published results are difficult to interpret, with large age discrepancies and/or failure to report ages. Age-matching becomes even more significant in studies of fetuses (de Lacoste et al., 1986; Clarke et al., 1989) and children (Bell and Variend, 1985; Clarke et al., 1989) when significant age-related changes in the CC may occur over weeks or months (Witelson and Kigar, 1988).

Apart from differences in actual measurements of the CC and differences in age distribution, discrepancies in reports of CC sex differences may arise from rel-

atively small sample sizes, differences in the general health of subjects, limited resolution of an MRI relative to a photograph of a postmortem section, and possible failure to obtain a precise midsagittal section of the CC. Furthermore, factors for which we were unable to account, such as genetic and racial constitution, handedness of the subject (Witelson, 1985, 1989; Nasrallah et al., 1986), and the environment (rats, Berrebi et al., 1988; Juraska and Kopcik, 1988) may influence the shape and size of the CC.

Further discrepancies in the literature may arise when multiple measurements of the CC are taken. With respect to this study, a number of evaluations of the shape of the CC, including subjective evaluation, MSW, and several bulbosity coefficients, consistently demonstrated a significantly more bulbous splenium in the CC of females; therefore, we feel confident that this difference exists. In contrast, a total of 23 measurements were made of areas of the CC and its subdivisions. We would expect by chance at the level of  $p < 0.05$  that one of these areas would exhibit a sex difference. Indeed P2 – P3 was significantly larger in males. Similarly, Witelson (1989) exhibited a sex-by-handedness interaction in this region when the CC was examined by the S-L method, which originally motivated us to use this method in our study. However, following the conservative Bonferroni adjustment for multiple comparisons, we found no significant sex difference in P2 – P3. Therefore, it is uncertain whether there are sex differences in the area of the P2 – P3 region, or whether the significance we obtained is a result of multiple comparisons. Interestingly, a larger P2 – P3 in males tends to make the splenium appear more bulbous in females.

#### **When Does Sexual Differentiation Occur?**

Although our sample size of children is small, it appears that sex differences in the shape of the CC may be present between the ages of 2 and 16 yr of age. The gender classification based on shape of the splenium was correctly identified in 1% more children than adults, and the percentage differences between bulbosity coefficients were greater between girls and boys than between women and men. However, neither the subjective gender classification nor the bulbosity coefficients reached statistical significance in children, perhaps because of the relatively small sample size. In contrast, the MSW was greater in boys than in girls, though it was greater in girls relative to CC area. However, a significantly greater MSW has been reported in female fetuses (de Lacoste et al., 1986).

#### **How Does Sexual Differentiation Occur?**

In the CC of the human being, it is not only unknown when sexual differentiation occurs, but also whether

the process of sexual differentiation is due to genomic factors, the environment, and/or gonadal hormone levels. However, in laboratory animals, nearly all sexually dimorphic structures exhibit morphological differences during the perinatal period. Furthermore, structures that are sexually dimorphic in rats, including the cerebral cortex (Diamond, 1988) and the CC (Berrebi et al., 1988; Juraska and Kopcik, 1988), are influenced by environmental factors both pre- and postnatally in a sexually dimorphic manner. For example, the sexually dimorphic pattern of cerebral cortical asymmetry may be influenced by both prenatal stress (Fleming et al., 1986) and an "enriched" postnatal environment (Diamond, 1988) and the CC may be influenced prenatally by maternal alcohol consumption (Zimmerberg and Scalzi, 1989) and postnatally by handling (Berrebi et al., 1988). However, more striking have been data indicating that nearly all sexually dimorphic structures examined thus far have been shown to be influenced by perinatal gonadal hormone levels.

The mechanism by which environmental factors influence neural structure in a sex-dependent manner is unclear. In contrast, gonadal hormones may act, at least in some cases, during a critical period of perinatal development to influence the survival of neurons in sexually dimorphic nuclei (Nordeen et al., 1985). Because there is a concentration of sex steroids in the cerebral cortex of the developing rat (Sandhu et al., 1986) and rhesus monkey (MacLusky et al., 1986) and an elimination of neurons and their axons during the perinatal period (Berlucchi, 1981), it is conceivable that gonadal hormones influence the number of axons coursing through the CC of humans; such axonal elimination may explain sex differences in the CC of humans (Witelson, 1985; Clarke et al., 1989). In addition, gonadal hormones appear to influence myelination: in rats, estradiol increases myelination (Curry and Heim, 1966), and 5- $\alpha$ -reductase, the enzyme that converts testosterone to dihydrotestosterone, is present in high concentrations in white matter (Celotti et al., 1987), suggesting that testosterone may also be involved in this process.

#### **Differences in Myelination, Number, or Arrangement of Axons?**

It is unknown whether sex differences in regions of the CC correspond to sex differences in myelination, in the numbers of fibers, or to a different arrangement of axons coursing through the CC, perhaps caused by other sexually dimorphic neuroanatomical structures. Several studies indicate that there is no correlation between the number of axons coursing through the CC and the size of the CC. For example, in the splenium of rats, there are significantly more axons in females; however, there are more myelinated axons in males,

resulting in a splenium that is not sexually dimorphic in terms of either area or MSW (Juraska and Kopcik, 1988). Similarly, in rhesus monkeys, there is approximately a 2-fold variation among animals between the density of axons and the midsagittal area of the CC, and there is no correlation between area of the CC and number of axons (LaMantia and Rakic, 1990).

Furthermore, the CC of children increase with advancing age: it is unlikely that this is caused by an increase in the number of callosal axons; rather, in the human there is protracted myelination of the CC after the first decade, and certain regions of the brain exhibit myelination throughout life (Yakovlev and Lecours, 1967).

### Functional Significance

Human males and females differ with respect to cerebral lateralization of neuropsychological function (for reviews, see McGlone, 1980; Beaton, 1985; Kimura 1987); furthermore, the midsagittal area of the CC may relate to cerebral specialization of function (Berrebi et al., 1988; Schmidt and Caparelli-Dàquer, 1989; Witelson, 1989). Some investigators propose that, in humans, the midsagittal area of the CC relates inversely to cerebral lateralization. For example, Witelson (1989) observed that regions of the CC that contain fibers connecting asymmetrical neural regions, such as the planum temporale (de Lacoste et al., 1985), were larger in individuals whose hand preference may indicate less cerebral specialization (Beaton, 1985). In contrast, in rats, greater lateralization may correspond to greater callosal size (Berrebi et al., 1988). In mice, the size of the CC directly relates to morphological asymmetry of the cerebral cortex: those animals with deficits in or the absence of a CC do not exhibit morphological cortical asymmetry, whereas animals with a normally developed CC do exhibit cerebral asymmetry (Schmidt and Caparelli-Dàquer, 1989). It is unknown whether sexually dimorphic neuroanatomical asymmetries, which occur in both rats (Diamond et al., 1983) and humans (Wada et al., 1975), underlie sex differences in functional asymmetry and/or correspond to the number of axons coursing through specific regions of the CC. However, if sexual dimorphism in the human CC is in fact related to a sex difference in the number or relative distribution of axons, then this difference may, in part, underlie sex differences in cerebral lateralization.

In contrast to structural sex differences in nuclei that are present in regions known to control reproductive function, such as the SDN-POA of the rat, whereby there are relatively dramatic sexual dimorphisms in terms of both structure and function, with generally little overlap between males and females, sex differences in regions not directly related to reproductive function,

such as the CC in terms of structure and cerebral lateralization in terms of function, are relatively subtle, with considerable overlap between males and females. Compared to the sexually dimorphic nuclei, many of which appear to underlie sexually dimorphic function, the significance of structural sex differences in regions not directly related to reproductive function is poorly understood.

### Conclusion

The controversy regarding sex differences in the CC may subside with studies using consistent measurements of large samples of healthy, age-matched subjects. Utilizing several criteria, we observed that the *shape* of the splenium (considered here to be P5) was consistently wider or more "bulbous" in females than in males. In contrast, among 23 measurements of the areas of the CC and its subdivisions, only 1 division, P2 – P3 examined by the C-L method, exhibited a significant sex difference, which would be expected by chance. However, none of these differences reached statistical significance in children. After the establishment of sex differences within the CC, MRI may be useful in demonstrating a relationship between sex differences in neuroanatomy and neuropsychological function, and the role of gonadal hormones and the environment in the process of sexual differentiation.

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The steroid hormone estradiol ( $E_2$ ) either is required for or significantly modulates many behaviors, including cognitive behaviors (1–9). Learning tasks that involve reference memory tend to be impaired by  $E_2$  (1, 7, 8). In contrast, low levels of  $E_2$  may facilitate working memory (5, 9). In women, changes in ability on visuospatial tasks and memory recall over the menstrual cycle have been documented (10, 11). Also, beneficial effects of estrogen replacement on cognition have been noted in normally aging women (12, 13) and in women suffering from dementia associated with Alzheimer's disease (14, 15).

How estrogen acts on learning and memory is not clear. Estrogen binding has been reported throughout the rodent brain, including the hippocampus and cortex (16). Both characterized nuclear estrogen receptors ( $ER\alpha$  and  $ER\beta$ ) are present in extrahypothalamic regions (17, 18). In addition, *in vitro* work suggests that estrogen acts in a nongenomic fashion in hippocampal tissues (19, 20). Mice lacking functional  $ER\alpha$  have severe deficits in several aspects of reproduction, including behavior (21–24). On the other hand,  $ER\beta$  knockout mice ( $ER\beta$ KO) are subfertile, and sexual behavior is normal (25, 26). Thus, there has been speculation that  $ER\beta$  regulates estrogen's nonreproductive functions in the central nervous system (21, 27, 28).

Here we describe our work on spatial learning in female  $ER\beta$ KO mice. When treated with doses of  $E_2$  that do not affect learning in wild-type (WT) littermates,  $ER\beta$ KO mice had impaired ability to escape from the Morris water maze. Interactions between  $ER\alpha$  and  $ER\beta$  may contribute to the learning response; to test the hypothesis that lack of  $ER\beta$  influences the level of  $ER\alpha$  protein, mouse brains were examined for  $ER\alpha$  immunoreactivity ( $ER\alpha$ -ir), with emphasis on the hippocampus (HIPP).

## Methods

### Animals

Female mice (ages 5–7 mos) were of mixed 129/J and C57BL/6J background. Subjects were generated by crossing heterozygotic mating pairs carrying a single

copy of the disrupted  $ER\beta$  gene (25). The resulting offspring were genotyped by PCR amplification of tail DNA. WT and  $ER\beta$  disrupted ( $ER\beta$ KO) littermates were used in these studies. The  $ER\beta$  gene disruption was created by Neo insertion into exon 3 (25), thus the following three primers were used: one from intron 2 (5'-GGAGTAGAAACAAGCAATCCAGACATC-3'), another from the 3' end of the Neo insert (5'-GCAGCCTCTGTTCCACATACACTTC-3'), and a third from exon 3 (5'-AGAATGTTGCACTGCCCCTGCTGCT-3'). A 665-bp band (intron 2 and exon 3 primers) was amplified for homozygous WT mice, a 500-bp band (intron 2 and Neo primers) for homozygous mutant mice, and both bands for heterozygous mice.

After surgery, mice were individually housed in polycarbonate cages, maintained on a 12:12-h light/dark cycle (lights off at 1800 h Eastern Daylight Time), and received food (Purina mouse chow no. 5001) and water *ad libitum*.

### Surgery and Hormone Administration

All mice (WT,  $n = 25$ , and  $ER\beta$ KO,  $n = 30$ ) were ovariectomized (OVX) under ketamine/xylazine anesthesia (20/2 mg per 25 g body weight). At time of surgery, each female was randomly assigned to a treatment group and received either an  $E_2$  17 $\beta$ -filled or an empty Silastic implant (controls).  $E_2$  implants were made in Silastic tubing via two methods. One type of implant was made by packing 5 mm of crystalline 17 $\beta$ - $E_2$  into Silastic tubing (i.d. 1.02  $\times$  o.d. 2.16 mm). We anticipated that this would yield a relatively high dose of  $E_2$  in plasma. The other implants were produced by first dissolving 17 $\beta$ - $E_2$  in sesame oil vehicle (2.5  $\mu$ g/0.025 ml) and then infusing 0.025 ml into a Silastic implant (i.d. 1.02  $\times$  o.d. 2.16 mm). Both ends of the Silastic tubing were glued with adhesive. Dilution of  $E_2$  in sesame oil was done to create a relatively low concentration of  $E_2$  in plasma. Thus a total of six groups were formed with 8–13 individuals per group. Silastic implants were administered *s.c.* and were placed in the midscapula region.

Ten days after surgery, each animal was tested for behavior by observers that were uninformed as to the

genotype of the animals. Three to four days after behavior testing was concluded mice were deeply anesthetized with sodium pentobarbital (10 mg/kg) and quickly decapitated. Blood was collected and spun, and plasma frozen for E<sub>2</sub> assay. Brains were rapidly removed and immersion fixed in 5% acrolein. Uteri were removed, cleaned of fat and connective tissue, and weighed. Animal care was conducted in accordance with the University of Virginia Animal Care and Use Committee guidelines.

### Water Maze Training

Our procedures have been described in detail (8). Briefly, each mouse was tested over 4 consecutive days before lights off during the lighted portion of the light/dark cycle. Animals were trained in a black circular pool (120 cm in diameter) located in a lit room containing a number of two- and three-dimensional visual cues. Pool water was maintained at  $23 \pm 2^\circ\text{C}$ . A black escape platform (10.5 cm in diameter) was submerged 1.5 cm below the surface of the water. The location of the platform remained the same throughout the 4-day training period.

On the first day, each mouse was given a 30-sec free swim and then assisted to the platform where she remained for a 30-sec rest. This pattern was repeated four times, once from each equidistant release site. Training consisted of three blocks of four trials each for 4 consecutive days. Each trial lasted for 60 sec or ended sooner if the mouse reached the submerged platform, thus escaping from the swim maze. If a mouse failed to find the platform in 60 sec, she was assisted to it. Between trials, each mouse rested on the platform for 20 sec. Mice were released in random order from one of the four release sites. Data collected for each trial were: latency to escape from the water maze (find the submerged escape platform), and whether the mouse succeeded in finding the platform at all during the 60-sec trial.

After data were collected and analyzed for the spatial version of the task, a separate group of five ER $\beta$ KO mice were run on the cued version. In this test, the escape platform is 5 cm above the surface of the water and is clearly marked with a large white flag. All competing visual cues in the room are removed. The rationale for this test is that mice that failed to find the escape platform in the spatial test should be able to locate the platform when it is visible. Because this task is relatively simple to learn, it is conducted only over a 3-day interval.

### Immunocytochemistry

Because the largest behavioral effects were noted between the control females and those in the high E<sub>2</sub> dose group, we limited the histological analysis to

brains collected from these animals (both genotypes,  $n = 6\text{--}11$  per group). Brains fixed in 5% acrolein were cryoprotected overnight in 30% sucrose at  $4^\circ\text{C}$  and quickly frozen by using 2-methyl butane cooled in dry ice. Brains were stored at  $-70^\circ\text{C}$  until they were sectioned.

Frozen brains were sectioned coronally at 30  $\mu\text{m}$  and divided into a series of three wells. One well of tissue (one-third of the sections collected) was processed by using a rabbit antiserum specific to ER $\alpha$  (C1355) made against the last 14 amino acids of the C-terminal region of the ER $\alpha$  protein (29). There is no homology between this region of ER $\alpha$  and - $\beta$ , respectively, thus there is no crossreactivity with ER $\beta$ . We have validated the use of this antiserum in mouse brain previously (30).

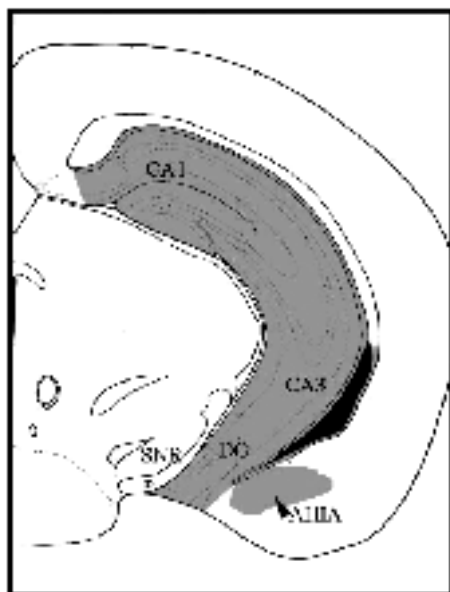
Immunoreactivity was visualized by using the Vector Elite ABC method (Vector Laboratories). Our methods have been described in detail (30). Nickel intensified diaminobenzidine (DAB) solution (0.25% nickel ammonium sulfate/0.05% DAB), activated by 0.001% hydrogen peroxide was used as the chromogen. Sections were rinsed, mounted onto gelatin coated slides, dehydrated, and coverslipped.

### Immunocytochemical Data Analysis

An observer uninformed as to the genotype and treatment of the animals scored the tissues. Immunoreactive cells were visualized by using an Olympus (New Hyde Park, NY) BX60 microscope attached to an Optronics charge-coupled device camera. The image analyses were conducted with METAMORPH software (Universal Imaging, Media, PA). The number of ER $\alpha$ -ir neurons was counted. The images were captured and saved for each animal from unilateral matched sections of each brain by using well-defined landmarks and a mouse brain atlas (figure 54 of ref. 31; interaural coordinate = 1.00 mm, Bregma =  $-2.80$  mm). Absolute cell counts and counts per microns squared were made. The landmarks include the shape and size of the lateral ventricles and the cerebral peduncle and the periaqueductal gray. Two regions were quantified in the same section. The regions included the total dorsal-to-ventral extent of the dentate gyrus, CA1–3 in the hippocampus (referred to as HIPP) and the amygdalohippocampal area (AHIA; figure 72.1).

### E<sub>2</sub> Assay

All samples were assayed in singlicate in a single assay. A commercial assay kit (Ultra-Estradiol, Diagnostic Systems Laboratories, Webster, TX) was used. The theoretical sensitivity of the assay is 0.6 pg/ml, and the standard curve ranges from 1.5 to 150 pg/ml. Maximum binding was 28%, and intraassay variability was 10.4%.



**Figure 72.1**

Cameralucida representation of the section we selected for the ER $\alpha$ -ir cell quantification, adapted from ref. 31 (figure 54; interaural coordinate = 1.00 mm, Bregma = -2.80 mm). The extensive shading dorsal to ventral that includes the CA1-3 and dentate gyrus represents the region of the hippocampus in which ER-ir cells were counted. Lateral to this area the small shaded region represents the AHIA. CA1, CA1 field of hippocampus; CA3, CA3 field of hippocampus; SNR, reticular substantia nigra; cp, cerebral peduncle.

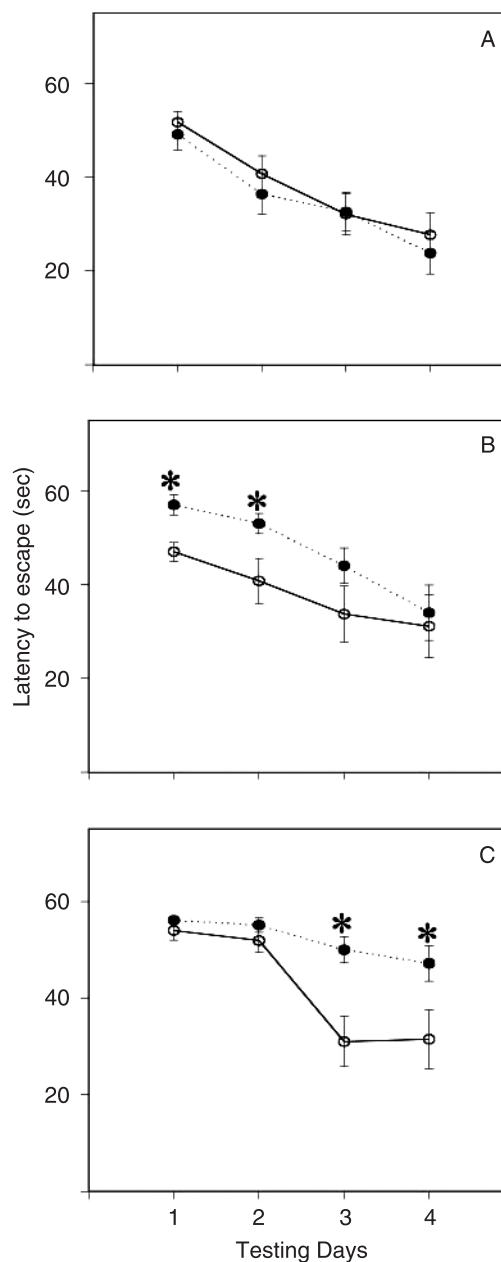
### Statistics

All behavior data were analyzed by repeated-measures ANOVA by using genotype, E<sub>2</sub> dose, and test day as the treatment factors. Specific genotype differences were analyzed over time and on specific test days with two-way ANOVA. We conducted the behavioral analyses on the average escape latency per day for each subject. In addition, we calculated a success score based on the number of times each day the mouse was able to locate the platform during each 60-sec trial. The maximum score was 12, and the minimum was 0. The immunocytochemical data were subjected to three-way ANOVA. The three factors were: immunocytochemical run (sections were developed in two runs separated by several months), genotype, and hormone treatment. E<sub>2</sub> concentrations in plasma and uterine weights were analyzed via two-way ANOVA. Bonferroni's planned comparisons corrected for multiple comparisons were conducted to assess group differences.

### Results

#### E<sub>2</sub> Treatment Impedes Escape Behavior in ER $\beta$ KO Mice

OVX ER $\beta$ KO mice treated with E<sub>2</sub> escaped from the Morris water maze more slowly than WT littermates (figure 72.2). A two-way repeated-measures ANOVA revealed significant effects of hormone



**Figure 72.2**

Changes in latencies (group means  $\pm$  SEM) to reach the escape platform in the Morris water maze over the 4-day training period. Data from WT mice are represented by open circles and solid lines. ER $\beta$ KO data are represented by closed circles and dashed lines. In A, data from OVX mice ( $n = 8$  WT,  $n = 9$  ER $\beta$ KO) are compared. B features data from OVX control mice receiving a low dose of E<sub>2</sub> ( $n = 9$  WT,  $n = 8$  ER $\beta$ KO). Finally, in C, data from females receiving the higher dose of E<sub>2</sub> are shown ( $n = 8$  WT,  $n = 13$  ER $\beta$ KO). \*, significantly different from WT females (comparisons on the same day).

dose [ $F(2, 220) = 5.60$ ,  $P < 0.0065$ ] and genotype [ $F(1, 220) = 4.47$ ,  $P < 0.04$ ] on latency to find the submerged escape platform. When control OVX WT and ER $\beta$ KO mice were compared, no differences were found in escape latencies for any of the testing days [ $F(1, 17) = 0.54, 0.54, 0.01$ , and  $0.36$  on days 1, 2, 3, and 4, respectively]. However, when females that received E<sub>2</sub> were examined, an effect of genotype was found [ $F(1, 152) = 9.23$ ,  $P < 0.005$ ], as well as a three-way interaction between genotype, hormone dose, and test day [ $F(3, 152) = 4.06$ ,  $P < 0.01$ , figure 72.2].

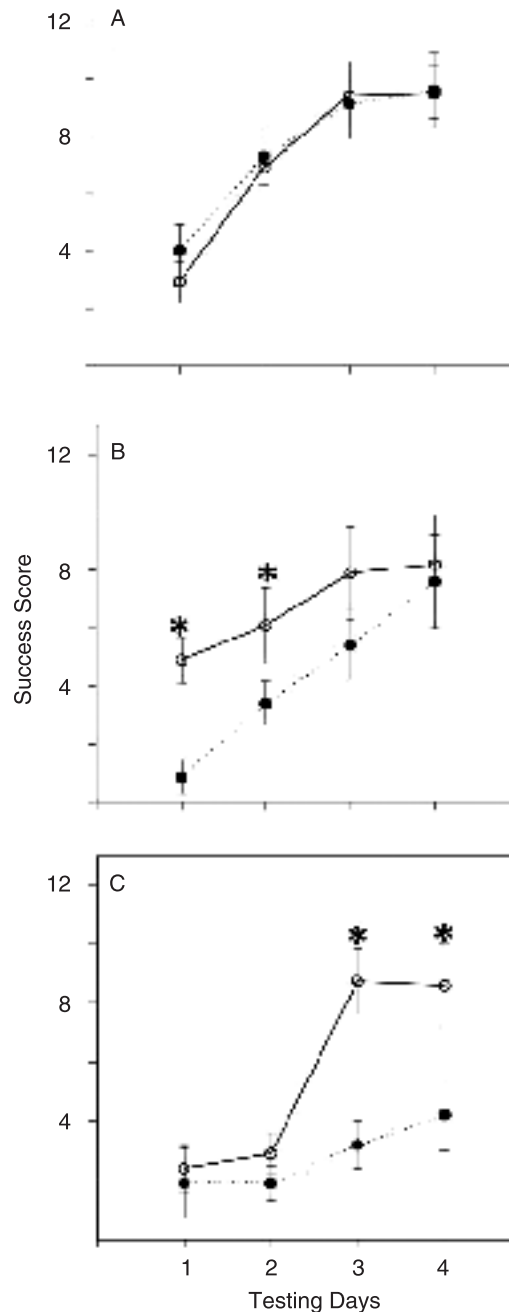
WT mice given the low dose of E<sub>2</sub> were faster to escape than ER $\beta$ KO animals on test days 1 [ $F(1, 17) = 11.42$ ,  $P < 0.005$ ] and 2 [ $F(1, 17) = 4.92$ ,  $P < 0.045$ ]. By the last 2 test days, no differences between the genotypes were noted [ $F(1, 17) = 1.97, 0.10$  on days 3 and 4, respectively]. In contrast, in the high E<sub>2</sub> dose group significant differences were noted on the final 2 testing days [ $F(1, 21) = 12.74$ ,  $P < 0.002$  on day 3 and  $F(1, 21) = 5.35$ ,  $P < 0.035$  on day 4]. Again, ER $\beta$ KO mice took longer to find the platform and thus escaped from the maze more slowly compared with WT mice. No such differences existed during the first 2 test days [ $F(1, 21) = 1.38, 1.33$  for days 1 and 2; figure 72.2].

Among WT females, no differences in escape latencies were attributed to hormone treatment [ $F(2, 100) = 0.36$ ]. However, latencies to escape depended on E<sub>2</sub> treatment in ER $\beta$ KO females [ $F(2, 120) = 12.37$ ,  $P < 0.0002$ ], and an interaction between dose and test day was noted [ $F(6, 120) = 2.62$ ,  $P < 0.025$ ]. ER $\beta$ KOs receiving the higher dose of E<sub>2</sub> were slower to escape on days 2, 3, and 4 compared with untreated OVX ER $\beta$ KOs ( $P < 0.05$ ). In addition, ER $\beta$ KOs that received the lower E<sub>2</sub> dose escaped more slowly than untreated females on day 2 and faster than females in the high E<sub>2</sub> group on day 4 ( $P < 0.05$ ).

#### E<sub>2</sub> Inhibits Successful Location of the Escape Platform in ER $\beta$ KO Mice

Analysis of success scores yielded a pattern of results that mirrors the escape latency findings. The ANOVA revealed significant effects of hormone dose [ $F(2, 220) = 6.65$ ,  $P < 0.003$ ] and genotype [ $F(1, 220) = 5.49$ ,  $P < 0.025$ ], and no interaction between these variables was detected. When scores for OVX control mice were examined, improvement was noted over time [ $F(3, 67) = 26.60$ ,  $P < 0.00001$ ], but there were no effects of genotype [ $F(1, 67) = 0.10$ ]. Scores from females treated with E<sub>2</sub> were influenced by genotype [ $F(1, 152) = 9.15$ ,  $P < 0.005$ ]. A three-way interaction between hormone dose, genotype, and testing day [ $F(3, 152) = 5.81$ ,  $P < 0.001$ ; figure 72.3] was noted.

On days 1 and 2 of testing, ER $\beta$ KO mice treated with the low E<sub>2</sub> dose were less successful at locating



**Figure 72.3**

Changes in success scores (group means  $\pm$  SEM) in the Morris water maze over the 4-day training period. All mice were OVX. Data from WT mice are represented by open circles and solid lines. ER $\beta$ KO mouse data are represented by closed circles and dashed lines. In A, data from OVX mice are compared. B features data from OVX mice receiving a low dose of E<sub>2</sub>. Finally, in C, data from females receiving a high dose of E<sub>2</sub> are shown. \*, significantly different from ER $\beta$ KO females (comparisons on the same day).

the escape platform than WT littermates treated with the same dose [ $F(1, 17) = 11.40, 4.92$  respectively;  $P < 0.043$ , at least]. No differences were present on days 3 and 4 [ $F(1, 17) = 1.97$  and  $0.75$ , respectively]. On the last 2 testing days, OVX ER $\beta$ KO mice treated with the high dose of E<sub>2</sub> were less successful than WT littermates given the same E<sub>2</sub> dose [ $F(1, 21) = 12.74, 5.35$ , respectively;  $P < 0.034$  at least; figure 72.3].

In WT females, an interaction between hormone dose and test day was noted [ $F(6, 100) = 3.19, P < 0.01$ ]. On test day 2, WT females treated with the high E<sub>2</sub> dose had lower success scores than OVX females. Both hormone dose effects [ $F(2, 120) = 11.53, P < 0.00025$ ] and an interaction between test day and dose [ $F(6, 120) = 2.27, P < 0.045$ ] were noted in ER $\beta$ KO females. On test days 2, 3, and 4, females in the high E<sub>2</sub> group had significantly lower success scores than OVX control females ( $P < 0.05$ ).

#### E<sub>2</sub> Does Not Inhibit Escape in the Cued Water Maze Task

To assess motor ability and general motivation to exit the water maze, we conducted a cued test with five ER $\beta$ KO individuals. All were OVX and treated with the high E<sub>2</sub> dose, as described in Methods. Females displayed a significant decrease in latency to find the platform [ $F(2, 15) = 21.83, P < 0.0006$ ] and an increase in success scores [ $F(2, 15) = 11.17, P < 0.005$ ] over the 3 testing days. In both measures, the differences lie between the first test day and the other 2 days ( $P < 0.05$ ). By the second day of testing, females found the platform in less than 20 sec and were successful in their escape attempts 11 of 12 times.

#### Estrogen Treatment Affects Uterine Weights and Concentrations of E<sub>2</sub> in Plasma

Hormone treatment, but not genotype, had a significant effect on uterine weights [ $F(2, 64) = 47.99, P < 0.000001$ ]. Uterine weights in each treatment group differed significantly from the other two groups ( $P < 0.05$ ). The means (in milligrams) and SEM for uteri collected from females in each hormone group were as follows: OVX =  $24.56 \pm 2.37$ ; OVX + E<sub>2</sub> oil-diluted implant =  $88.12 \pm 13.30$ ; OVX + crystalline E<sub>2</sub> implant =  $197.33 \pm 12.44$ .

In addition, hormone treatment had the desired effect of creating different concentrations of E<sub>2</sub> in plasma [ $F(2, 51) = 46.62, P < 0.000001$ ]. The crystalline E<sub>2</sub>-17 $\beta$ -packed implant yielded a blood concentration that averaged  $98.05 \pm 6.00$  (SEM) pg/ml. This concentration is similar to that measured in plasma of C57BL/6J mice during proestrous (ref. 32; E.F.R., unpublished data). This E<sub>2</sub> concentration differs significantly from the measured plasma levels in OVX controls ( $16.1 \pm 9.3$  pg/ml) and in OVX mice that received the oil-diluted dose of E<sub>2</sub> ( $13.4 \pm 3.6$  pg/ml).

**Table 72.1**

Numbers (mean  $\pm$  SEM) of immunoreactive ER $\alpha$  cells in the AHIA and hippocampus

	AHIA	Hippocampus
WT OVX	$235.5 \pm 30.0$	$150.0 \pm 18.0$
WT OVX + E <sub>2</sub>	$235.0 \pm 20.5$	$158.0 \pm 17.6$
ER $\beta$ KO OVX	$202.0 \pm 21.0$	$149.0 \pm 24.0$
ER $\beta$ KO OVX + E <sub>2</sub>	$211 \pm 17.7$	$93.4 \pm 13.0^*$

\* Significantly different from hippocampus ER $\alpha$ -ir cell numbers in ER $\beta$ KO OVX females ( $P < 0.05$ ).

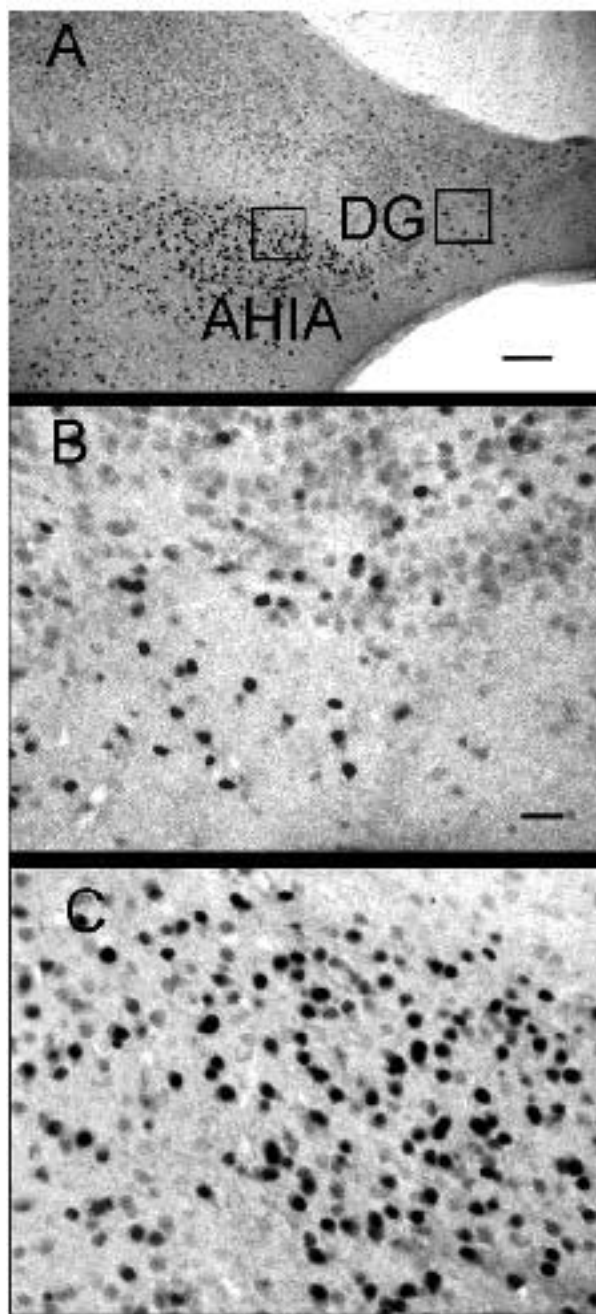
It is clear from behavioral data and uterine weights that control OVX females and OVX mice treated with the low dose of E<sub>2</sub> were experiencing different levels of E<sub>2</sub> in the blood. However, because the low E<sub>2</sub> dose yields plasma E<sub>2</sub> concentrations close to the bottom end of the physiological range, we are not surprised that the assay did not show a significant difference in plasma E<sub>2</sub> levels among females in these groups. Moreover, this concentration (13.4 pg/ml) is similar to that measured during diestrus in ovary-intact mice (32).

#### ER $\beta$ Affects Expression of ER $\alpha$ -ir in Hippocampus

Decreased numbers of ER $\alpha$ -ir cells were noted in hippocampi of estrogen-treated ER $\beta$ KO females compared with untreated OVX ER $\beta$ KO mice [ $F(1, 32) = 5.82, P < 0.025$ ] or any other treatment group tested. In the hippocampus, an effect of hormone treatment was noted on ER $\alpha$ -ir cell numbers. There was no interaction between the effects of hormone treatment and immunocytochemical run [ $F(1, 32) = 0.01$ ], nor was there an effect of genotype [ $F(1, 32) = 1.44$ ]. The effect of hormone treatment on ER $\alpha$ -ir can be attributed to significantly fewer ER $\alpha$ -ir cell numbers in brains of ER $\beta$ KO females that received the high E<sub>2</sub> dose as compared with OVX ER $\beta$ KO mice ( $P < 0.05$ ; table 72.1). In the AHIA, no significant effects of immunocytochemical run, hormones, or genotype were noted [ $F(1, 36) = 0.01, 2.10, 0.00$ , respectively]. Intensely stained ER $\alpha$ -ir cells were present in the AHIA (figure 72.4). In the hippocampus, many lightly stained cells were present throughout the region, but only the darkly immunoreactive cells, similar in intensity to those counted in the AHIA, were counted (figure 72.4). Most of these ER $\alpha$ -ir cells were noted in the ventral portion of the granular layer of the dentate gyrus.

#### Discussion

Our data show that two doses of E<sub>2</sub>, which yield plasma concentrations similar to those experienced during the mouse estrous cycle, either completely blocked (high dose) or delayed (low dose) learning acquisition in the spatial water maze task in ER $\beta$ KO but not in WT



**Figure 72.4**

Photomicrographs of ER $\alpha$ -ir in the hippocampus and adjacent AHIA. A low-magnification view is presented in *A* (20 $\times$ ) and even higher ( $\times 40$ ) examination of ER $\alpha$ -ir cells in the hippocampus (*B*) and AHIA (*C*). Boxed regions in *A* represent the areas shown in *B* and *C*. (Bar in *A* = 100  $\mu$ m; in *B* = 10  $\mu$ m.) DG, dentate gyrus; AHIA, amygdalohippocampal area.

littermates. Thus, the lack of ER $\beta$  severely impairs spatial learning in mice. Because the doses of E<sub>2</sub> were within the physiological range, we infer that ER $\beta$  is actively involved in reference memory formation during the normal estrous cycle. In contrast, our previous work with an even higher dose of E<sub>2</sub> than used here showed that water maze learning was impaired in WT but not in ER $\alpha$ KO females (8). Together, these data suggest that the inhibitory effect of E<sub>2</sub> on learning is mediated by ER $\alpha$ , and the lack of ER $\beta$  increases sensitivity to the negative consequences of E<sub>2</sub> on reference memory, particularly spatial learning. Thus, when ER $\beta$  is not functional, the actions of E<sub>2</sub> on ER $\alpha$  are "unmasked" and are more pronounced than under conditions where both ERs are responsive.

One way to describe this relationship is to examine E<sub>2</sub> dose-dependent effects on behavior in WT and ER $\beta$ KO females. In WT females, there was only a single time point when a difference in behavior could be attributed to concentrations of E<sub>2</sub> in plasma. On day 2, success scores for OVX WT mice were significantly higher than scores for females in the high E<sub>2</sub> dose group. In contrast, ER $\beta$ KO females treated with the high E<sub>2</sub> dose had lower success scores than their OVX counterparts on all except the first day of the task. In addition, in ER $\beta$ KO but not in WT females, there were significant effects of E<sub>2</sub> dose on latencies to escape from the maze. Slower escape latencies accompanied poor success scores in females receiving the high E<sub>2</sub> dose. The low E<sub>2</sub> dose also had behavioral effects in ER $\beta$ KOs; however, these effects were transient. On the first 2 days of testing, ER $\beta$ KO females in the low E<sub>2</sub> group were slower to escape than untreated OVX mice. On the last day, ER $\beta$ KOs in the low E<sub>2</sub> group were faster to escape than their counterparts in the high E<sub>2</sub> dose group. Thus, in the absence of ER $\beta$ , treatment with a low dose of E<sub>2</sub> delayed spatial learning, whereas exposure to the high E<sub>2</sub> dose blocked learning acquisition during the 4-day test. Importantly, these dosage effects of E<sub>2</sub> within the physiological range are apparent only in the absence of functional ER $\beta$ .

ER $\beta$  may also serve to protect ER $\alpha$  protein from down-regulation by E<sub>2</sub>, perhaps by binding available ligand. Here we show that the numbers of ER $\alpha$ -ir-positive nuclei after estrogen treatment were significantly reduced only in ER $\beta$ KO mice. Likewise, in regions of the hypothalamus such as the ventromedial nucleus, preoptic area, and the arcuate nucleus in ER $\beta$ KO, but not in WT females, this same down-regulation of E<sub>2</sub> on ER $\alpha$ -ir cells has been reported (33). In the hippocampus, reduced ER $\alpha$  could influence the levels or activity of many growth factors and/or neurotransmitters known or suspected to be involved in learning, including acetylcholine, neural growth factors, and their receptors (34–37).

Several studies have documented direct interactions between ER $\alpha$  and - $\beta$  both in vitro (38–40) and in vivo (33, 41, 42), and colocalization of the two receptor forms occurs in specific subsets of neurons throughout the brain (43, 44). ER $\alpha$  and - $\beta$  can form heterodimers in vitro and, in cell transfection studies, ER $\beta$  functionally suppresses ER $\alpha$  transcriptional activity (39, 45–47). ER $\beta$  has been postulated to act as a repressor to ER $\alpha$  in complicated processes such as cell growth or tumorigenesis, and changes in the ER $\alpha$ /- $\beta$  ratio are associated with several types of cancer (48, 49). The mechanism of estrogen action on learning is unknown but could include both genomic and nongenomic effects as well as actions mediated via other proteins or receptors. For any of these pathways, ER $\beta$  might act as a repressor of ER $\alpha$ . For example, E<sub>2</sub> activates the mitogen-activated protein kinase (MAPK) cascade within minutes in cortical tissue (20) and facilitates rapid initiation of kainate-induced current in individual hippocampal neurons (19). Neither effect was blocked by the potent antiestrogen ICI 182,780. Moreover, when brain tissues from WT and ER $\alpha$ KO mice were compared, extracellular signal-regulated kinases (ERK) phosphorylation was significantly enhanced in the absence of functional ER $\alpha$  (20). Thus, ER $\alpha$  may suppress overall MAPK activity, either directly or indirectly. Recently, a G protein-coupled receptor homolog, GPR30, has been shown to affect ERK activation in the absence of either ER $\alpha$  or - $\beta$ , perhaps via a nongenomic action of E<sub>2</sub> on growth factors (49). It is possible that E<sub>2</sub> actions on the MAPK cascade may have consequences for learning when both ER $\alpha$  and - $\beta$  are present, and these actions may be disrupted when one or both of the ERs is not available.

Another profound manner in which estrogens could affect learning and memory is via neural remodeling and plasticity. E<sub>2</sub> alters many aspects of hippocampal morphology, including synapse number, spine density, and astrocytic volume (50–52). In brain, astrocytes contain ER $\beta$  (53, 54). Moreover, ER $\beta$ KO brains display fewer Nissl-stained neurons and increased glial fibrillary acidic protein immunoreactive cells in the medial amygdala and preoptic area, as compared with WT littermates (55). It is well known that adult neurogenesis can be triggered by estrogen (56), yet the ER responsible for these effects has not been identified. In addition, estrogen exposure can promote or deter apoptosis (57), with the identity of the ER responsible for neuroprotection under debate (58, 59). Regardless of whether ER $\beta$  acts to reduce estrogen-related synaptogenesis, glia concentrations, prevent cell death and/or stimulate neurogenesis, any or some of these effects may impact learning and memory.

Learning is a complex behavior, and it is possible that another behavioral phenotype of the ER $\beta$ KO

mice could affect their ability to learn. Sexual behaviors have been reported to be normal in male and female ER $\beta$ KO mice (26). In addition, although ER $\alpha$ KO females required estrogen treatment to learn to avoid shock in a simple 24-h learning task, WT and ER $\beta$ KO females performed well on the task regardless of estrogen status (60). Yet, one recent report suggests that female ER $\beta$ KO mice have elevated levels of anxiety and reduced levels of activity in open field tests (61). We have collected similar data from OVX ER $\beta$ KO and WT female littermates given no hormone or treated with the high E<sub>2</sub> dose used in the current experiments (D. B. Imwalle and E.F.R., unpublished data). In our study, ER $\beta$ KO mice displayed elevated anxiety, but E<sub>2</sub> had no effect. Thus, although anxiety could be a contributing factor, it cannot completely explain the E<sub>2</sub> dose-dependent learning impairment in ER $\beta$ KOs.

Our results are compelling: WT females were able to learn a spatial task with or without E<sub>2</sub> replacement, but ER $\beta$ KOs could perform only when no E<sub>2</sub> was given. Yet there are many issues that still need to be addressed. For example, in female rats, E<sub>2</sub> influences learning strategies (62). Thus it is still possible that lack of ER $\beta$  specifically influences a type or component of learning in a spatial task. Given our previous findings that ER $\alpha$ KO females do not learn well in an inhibitory avoidance task (60), it is tempting to speculate that the two ERs affect different types of learning; perhaps ER $\alpha$  is needed for emotional learning that is likely to rely on the amygdala, whereas hippocampal-dependent spatial learning requires ER $\beta$ . Given the demographics of aging and increase in treatment of menopausal symptoms with estrogen replacement therapy, it would be useful to know more about the interactions and independent functions of the two ERs. These data can lead to the development of ER-specific agonists and antagonists that will be needed to ensure proper cognitive function in postmenopausal women.

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## 1 Introduction

The aromatization hypothesis [1] posits that the brain of the developing male rodent is masculinized by the activity of estrogen receptors, following the conversion of testosterone (T) into 17- $\beta$  estradiol (E<sub>2</sub>) by the enzyme aromatase. While most of the support for this hypothesis has come from examinations of sexual behaviour and morphological changes in the hypothalamus following hormone application neonatally [2–6], other work examining non-reproductive behaviours has also been found to fit within this framework [7, 8].

Spatial reference memory is a sexually dimorphic cognitive ability in which males are often found to outperform females [9–14] on a variety of different tasks, including the Morris Water Maze (MWM). Neonatal castration of males leads to a female-typical pattern of performance in several different MWM paradigms measuring this ability, while the application of testosterone to females early in development leads to a male-typical performance in adulthood [10, 15]. Similarly, work using the Radial Arm Maze (RAM) has shown that the neonatal application of estrogen to females results in a male typical pattern of performance in adulthood [7]. Given that the neonatal administration of the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD) seems to diminish adult male ability in adulthood [8], and that the castration of normal males in adulthood does not affect their performance [13, 14, 16], it would seem that the aromatization of T is responsible for the organization of this ability during some critical period immediately after birth.

Challenges to this hypothesis have emerged, however. A pair of experiments using the MWM have found that E<sub>2</sub> administration to females does not have any masculinizing effect on spatial reference memory; these authors suggest, instead, that the organization of this behaviour is accomplished by dihydrotestosterone (DHT), a non-aromatizable androgen that is likely acting through the androgen receptor (AR) [13, 14]. Convergent evidence comes from the findings that males administered the AR antagonists flutamide [13] or

cyproterone [16] prenatally perform at a female-typical level, and worse than normal males, on spatial reference memory tasks, as do males administered flutamide in adulthood [17]. Taken together, this work suggests that the AR may play more of a role in the organization (and activation) of spatial reference memory than was previously believed.

A novel way of approaching this issue is through the use of animals which lack functional AR. The testicular feminization mutation (*tfm*) is a single base-pair mutation of the gene encoding the AR [18], rendering the AR protein non-functional. As a result, males carrying this X-linked gene are fully affected, and develop a female phenotype. However, *tfm*-affected males develop secretory testes [19], and serum T levels are consistent with those of normal male littermates [19, 20], so the aromatization hypothesis would suggest that the conversion of T to E<sub>2</sub> should be sufficient to masculinize the brains of *tfm*-affected males.

This report is the first to examine the non-reproductive behaviours resulting from the testicular feminization mutation, and we are looking to determine if this is an appropriate model for clarifying the effects of AR activity on physiology and behaviour. In this study, we compared the performance of wild-type (WT) males and females with *tfm*-affected males and heterozygous female carriers of the mutation, using a hidden platform version of the water maze. We also performed a morphological analysis of the granule cell layer of the dentate gyrus (GCL-DG) in the hippocampus, a structure that is heavily implicated in spatial processing. Previous work has found that while two-dimensional measures in the dentate yield a sexual dimorphism [21, 22], a volumetric analysis failed to find any difference [13]. We wanted to see if we could replicate that lack of a difference, as well as begin to characterize the GCL-DG of *tfm*-affected males.

## 2 Methods

### 2.1 Animals

A total of 56 Sprague–Dawley (SD) rats between 70 and 80 days old were used for the water maze testing;

22 females, 22 males (XY), and 12 *tfm*'s ( $X^{tfm}Y$ ). Of the females, 8 were heterozygous for the *tfm* mutation ( $X^{tfm}X$ ), and 14 were WT, not carrying the mutation (XX). All animals used in this study were littermates bred in our *tfm* colony, group housed at the Simon Fraser University (SFU) Animal Care Facility (ACF), with access to rat chow and tap water ad libitum. The room temperature was held constant at 21 °C, and a 14:10 LD cycle was maintained, with lights on at 12:00 PM, noon. All behavioural testing was carried out at the ACF, and was approved by the SFU University Animal Care Committee, meeting the standards set forth by the Canadian Council on Animal Care.

## 2.2 Handling

All animals were handled for a total of 5 min per day for the 7 days preceding water maze testing, to reduce stress reactions resulting from handling during the testing period. During this time, animals were removed from their home cages, and placed on an elevated holding area [12], similar to the one on which they would be placed during testing. This procedure was used to allow the animals to habituate to daily handling by the investigator and to being on a similar type of the open, raised platform upon which they would be placed in between water-maze trials.

## 2.3 Testing Room and Apparatus

Testing occurred in a 1.5 m diameter pool centered within a rectangular room measuring  $4.7 \times 4.0$  m. An overhead camera was connected to a video monitor and a computer. Software (Chromotrack, San Diego Instruments, San Diego, CA) was used to track the animals' progress, and to calculate the time spent in each area of the pool. The water in the pool was made opaque by the addition of a nontoxic acrylic white paint. Water levels were 2.5 cm above the hidden platform, and maintained at  $23 \pm 1$  °C. A white, opaque curtain surrounded the pool, blocking access to major visuo-spatial cues and prominent landmarks within the testing room, and thereby increasing the difficulty of the spatial processing task. The use of a curtain has been described previously [12] and was used to here as it has been previously found to provide a male-typical sex difference in water-maze testing.

## 2.4 Water Maze Protocol

Each animal was tested four times a day for five consecutive days [12]. For scoring purposes, the pool was broken up into 4 equal sections, arbitrarily identified as northeast (NE), southeast (SE), northwest (NW), and southwest (SW). The platform was placed in the middle of the NE quadrant, for all testing. For some

scoring procedures, the pool was further divided into three equal-width rings: an outer ring (OR) immediately adjacent to the wall, an inner ring (IR) at the center area of the pool, and a middle ring (MR) lying between the OR and the IR. The animals were released into the pool from each of 4 starting locations daily, in a pattern that was randomly determined prior to testing. The starting points corresponded to north, east, west, and south, based on the position of the arbitrary quadrants discussed above.

Prior to each day of testing, the video recording device was calibrated and recording was started. For every trial, the animal was placed in the pool facing the wall. Animals were allowed 60 s to find the platform. If they were unable to find the platform in that time, they were guided to it by hand. They were allowed to remain upon the platform for 15 s, and were then removed. During testing, the investigator remained behind the curtains, so as to not act as a landmark cue. A minimum of 5 min elapsed between trials, during which time the animal was placed under a heat lamp, on an elevated platform in the testing room. All testing was started at noon, and the order in which the animals were tested was randomly changed, to prevent any time of day effects.

## 2.5 Identification of Females and *tfm*-Affected Males

For the duration of the testing period, the investigators were blind with regard to genotype across the three phenotypically female groups (XX,  $X^{tfm}X$ , and  $X^{tfm}Y$ ); genotype was identified after all testing was completed. Two procedures were employed for genotyping. Of the 34 phenotypic females, 18 were genotyped by breeding the females. Approximately 21 days following birth, pups were removed, and searched internally. Where breeding was unsuccessfully attempted on at least three separate occasions, the animal was also searched internally. A *tfm*-affected male was determined by the following criteria: phenotypic female genitalia, small urogenital distance, internal testes, and no inguinal canal. The presence of *tfm*-affected males in a litter indicated that the mother was a carrier of the mutation. In litters where there were no  $X^{tfm}Y$ , the mother was considered to be a WT female. The remaining 16 phenotypic females were identified through a polymerase chain reaction screen for the wild-type AR gene product [23]. This procedure was carried out at the Michigan State University (MSU), using a small sample of ear tissue collected under isoflurane anaesthesia.

## 2.6 Histology

After identification (at approximately 6 months old), 15 animals (6  $X^{tfm}Y$ , 5 XY and 4 XX, all age matched,

littermate controlled) were killed by CO<sub>2</sub>/O<sub>2</sub> inhalation. They were then perfused transcardially using 0.1M PBS followed by 4% paraformaldehyde (PFA), with a pH of 7.4. Brains were rapidly removed and immersed in PFA for a further 2 h postfix, followed by overnight immersion in a 20% sucrose solution (in PBS) as cryoprotection. Brains were sliced on a sliding microtome, 60 µm thick, and every fourth section was mounted, and Nissl stained using thionin. Slides were cover-slipped immediately with Permount (Fisher) and left overnight to dry.

## 2.7 Water Maze Analyses

A 3 × 5 mixed design (groups X day) repeated measures analysis of variance (RM ANOVA; SPSS v.11.5 for Windows) was used for examining the escape latency, speed, distance traveled, and the percentage of time spent in each of the four quadrants and three rings arbitrarily imposed upon the pool. The outer and middle rings were approximately 25 cm in diameter, whereas the inner ring was a circle with a diameter of 50 cm. Where overall *F*-tests were significant ( $p \leq 0.05$ ), individual two-tailed student's *t*-tests were used to compare the groups for each trial. The RM ANOVA was also used to examine the two female groups, WT-females, and carrier-females. A priori student's *t*-tests were used to compare the groups escape latency by day, as the aromatization hypothesis suggests that XY and X<sup>tfm</sup>Y group animals will perform better than the XX and X<sup>tfm</sup>X animals on this task. Finally, the number of direct swims was also examined using the RM ANOVA and Tukey's HSD post-hoc analyses. Direct swims were counted only when the animal swam in a straight line from the release point to the platform.

## 2.8 Morphological Analyses

The volume of the GCL-DG was estimated using MCID software (Imaging Research Inc., St. Catharines, Ont.) to reconstruct three-dimensional volumes from serial two-dimensional slices, by accounting for slice thickness and interslice interval throughout the extent of the structure. Subsequently, a one-way ANOVA was used to test for any group differences.

## 3 Results

With respect to escape latency, the overall RM ANOVA was significant for both the within subjects factors (trial session  $F_{19,1007} = 17.83$ ;  $p = 0.000$ ; Mauchly's  $W = 0.002$ , Greenhouse-Geisser corrected  $F = 17.83$ ,  $p = 0.000$ ) as well as for the between-subjects factors ( $F_{2,53} = 4.948$ ;  $p = 0.011$ ) (figure 73.1). This effect was not reducible to group differences

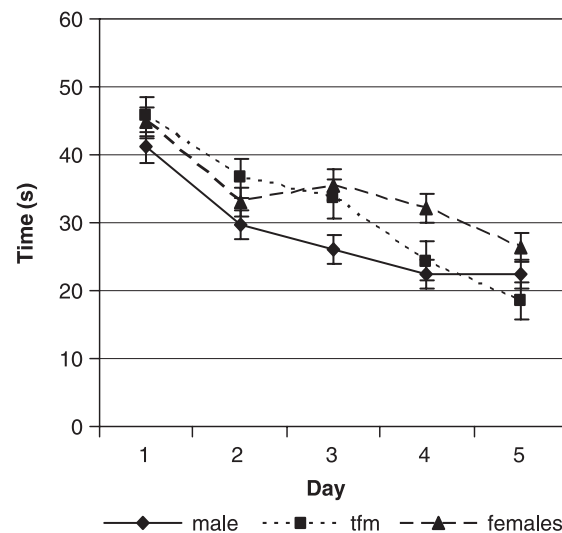
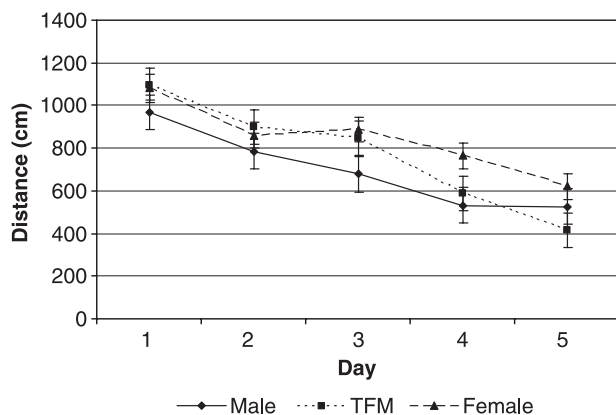


Figure 73.1

Morris water maze escape latencies. Means  $\pm$  S.E.M. for each of the three groups of animals (males, *tfm*-affected males, and females) by day.

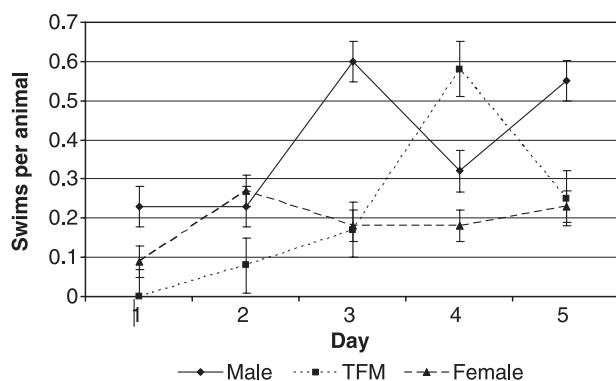
in speed ( $F_{2,53} = 0.056$ ;  $p = 0.945$ ). As the two female groups (XX and X<sup>tfm</sup>X) did not differ ( $F_{1,20} = 0.052$ ;  $p = 0.822$ ), they were collapsed together into one group, referred to as females. The XY group performed significantly better than females on days 3 and 4 ( $t_{174} = -3.19$ ,  $p = 0.002$ ;  $t_{174} = -3.292$ ,  $p = 0.001$ , respectively); by day 5, both groups were performing equally. The X<sup>tfm</sup>Y group showed an intermediate pattern of performance. On day 3, when the sex difference emerged, the X<sup>tfm</sup>Y group took significantly longer to find the platform than males ( $t_{134} = -2.117$ ;  $p = 0.036$ ), but not females. This pattern was reversed on day 4, where the X<sup>tfm</sup>Y group exhibited a male-typical performance, finding the platform significantly faster than the females ( $t_{134} = -2.139$ ;  $p = 0.034$ ). Intriguingly, the X<sup>tfm</sup>Y group also performed better than the females, but not males, on day 5 ( $t_{134} = -2.251$ ;  $p = 0.026$ ). An analysis of the individual trials on day 5 shows that this was due to a superior performance in the first two trials; on the last two trials, all three groups performed at identical levels, as expected (figure 73.1).

An analysis of the amount of time spent in the rings of the pool revealed no group differences in the time spent in the outer ring, middle ring or inner ring. This is interpreted here as indicating that there was no difference between groups on a commonly used measure of stress (thigmotaxis; discussed below). There was, however, a difference in the amount of time spent in the quadrants. Though no differences were observed in the target quadrant (NE), RM ANOVA did reveal a difference in the NW quadrant ( $F_{2,53} = 5.503$ ;  $p =$



**Figure 73.2**

Morris water maze distance traveled. Means  $\pm$  S.E.M. for each of the three groups of animals (males, *tfm*-affected males, and females) by day.

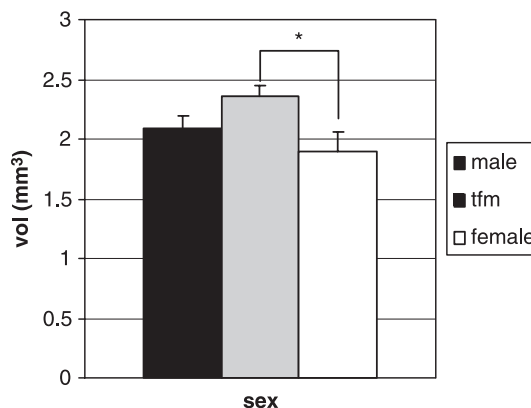


**Figure 73.3**

Morris water maze direct swims. Means  $\pm$  S.E.M. for each of the three groups of animals (males, *tfm*-affected males, and females) by day.

0.007), one of the incorrect adjacent quadrants. Specifically, males spent less time in this non-target quadrant than both  $X^{tfm}Y$  group and females on day 3 ( $t_{134} = -2.137$ ,  $p = 0.034$ ;  $t_{174} = -2.332$ ,  $p = 0.021$ , respectively), the day on which males performed better than both of these groups.

With respect to distance traveled, a similar pattern of performance was shown (figure 73.2). Again, we see the same pattern of performance as in escape latencies, and the RM ANOVA showed a significant difference for gender ( $F_{2,53} = 3.302$ ;  $p = 0.045$ ) as well as for day ( $F_{19,1007} = 12.995$ ,  $p = 0.000$ ; Mauchly's  $W = 0.002$ , Greenhouse-Geisser corrected  $F = 12.995$ ,  $p = 0.000$ ). The number of direct swims was also analyzed (figure 73.3). Again, the RM ANOVA showed that there was a significant difference in the number of direct swims ( $F_{2,53} = 3.910$ ;  $p = 0.026$ ), with males making more direct swims overall than both females, but not *tfm*'s, according to the post-hoc Tukey's HSD



**Figure 73.4**

GCL-DG volume estimations. Means  $\pm$  S.E.M. of the GCL-DG for each of the three groups of animals.

analysis ( $p = 0.028$ ). This difference was specific to the third day of training, the day upon which the sex differences in performance emerged.

The overall ANOVA for the GCL-DG volume suggested a difference across the three groups ( $F_{2,12} = 3.881$ ;  $p = 0.05$ ). Individual comparisons suggested that while there was no male–female difference, the  $X^{tfm}Y$  group had a significantly (24%) larger volume than the females ( $t_8 = 2.559$ ;  $p = 0.034$ ) (figure 73.4).

#### 4 Discussion

The results of the water maze study suggest that the *tfm*-affected male rats are only partially masculinized with respect to the acquisition of a spatial reference memory task, implicating androgen receptor activity in the male advantage often observed with this ability. We also have further replicated here the male superiority in the acquisition of this task.

It has been suggested that the differences commonly found on this task may not be due to spatial ability per se, but to a differential stress response across the two sexes that prevents the females from performing the task as adequately as males [12]. We found no evidence that this was a factor. First, the sex differences did not emerge until day three, beginning with the 9th trial that the animals were subjected to. One admittedly speculative possibility is that the amount of time until this difference emerged could have allowed for the females to adapt to the stress of the task, and until this time, all three groups, including females, still showed clear evidence of learning the maze to a certain point. Second, other researchers have suggested that females exhibit a stress-related behaviour termed thigmotaxis, in which they limit their exploration of this environment to the outer edge of the pool [24]. However, there were no group differences in this study with respect to the

amount of time spent in the outer ring of the pool, so it seems unlikely that the group differences in maze performance are reducible to thigmotaxis.

Consistent with previous reports, we found that the volume of the GCL-DG is not sexually dimorphic. Our data compared extremely well with those of the previous study [13], and showed excellent agreement in volume estimation for both the male and female groups (within 60  $\mu\text{m}^3$ ). This contrasts with earlier studies employing two-dimensional analyses, which are more inclined to report sex differences [21, 22]. Interestingly, though, we found a large difference between the  $X^{\text{tfm}}Y$  group and the females. The exact reason for this is unknown. Previous work characterizing the immunoreactivity of both AR and estrogen receptors (ER) in the hippocampus has been consistent in finding high levels of ER in the DG of both males and females [25–27]. However, while whole brain tissue homogenates from adult *tfm*'s have been found to contain normal male-typical levels of ER [28], ER-immunoreactivity has not been performed in discrete brain regions; thus, it is unknown how much ER is present in the DG of these animals. Given that androgens are also known to interact with the hypothalamic–pituitary–adrenal axis [29], and that glucocorticoids can affect the development of the hippocampus, it is possible that one of these two steroid systems may be altered as a result of the testicular feminization mutation, thus resulting in a slightly hypertrophic GCL-DG.

Some work in the rodent, specifically by Frye and her colleagues [30, 31], has noted an activational effect on specific cognitive tasks, however, these tasks have not included any measures of spatial navigation. Furthermore, research in the human has consistently found an activational effect of gonadal hormones on cognitive tasks, including spatial abilities [32, 33]. Studies employing the sexually dimorphic meadow vole (*Microtus pennsylvanicus*) have also been examined with respect to spatial reference memory, and have produced a similar male advantage on the Morris Water Maze [34, 35]. In particular, Galea and her colleagues noted that while female vole performance was strongly explained by estradiol level, with high levels of E2 associated with impaired performance, there were no activational effects of T apparent. Further studies also showed that the width of the dentate gyrus was affected by hormone titers; males with high T and females with high E2 showed increased DG area [36]. However, while hormone levels were measured in adulthood, it may be the case that these variations were also present during development. Previous works in the rat, however, have shown that castration in the adult male has no effect on spatial learning in different paradigms [13, 14, 16]. It is possible that a genuine

species difference exists with respect to the activational effects of gonadal hormones on spatial acquisition tasks. Thus, the sex difference here may be due, primarily, to the organizational effects of gonadal hormones.

The aromatization hypothesis cannot completely account for the results presented here; the AR must play a role in this behaviour, or the  $X^{\text{tfm}}Y$  group would perform exactly like the males in the MWM. It is possible that the lack of AR activity in the  $X^{\text{tfm}}Y$  animals is causing this intermediate pattern of performance as a result of the downregulation of aromatase, and thus, decreased estrogenic activity. However, we believe that this is not the case, for several reasons. Previous work has documented an androgen-insensitive “limbic-ring” of aromatase regulation [37]. Aromatase activity is androgen receptor dependent within the hypothalamus [38], but there is an androgen-independent regulation of this enzyme within areas of the limbic system, including the amygdala, lateral septum, and the bed nucleus of the stria terminalis [37, 39]. It is thus tempting to hypothesize that androgen-independent aromatase regulation occurs within the hippocampus as well, though this possibility has not yet been explicitly tested. Indeed, it has previously been shown that within the medial and cortical amygdala, there is no difference in aromatase activity between *tfm*-affected and control males [40], even though within the medial portion of this structure, aromatase activity is slightly sensitive to androgen levels [38]. This would suggest that the available T within the hippocampus of  $X^{\text{tfm}}Y$  group would be aromatized as per normal, and if typical ER activity occurs, then the difference in performance between males and  $X^{\text{tfm}}Y$  group must be due to AR effects outside of aromatase regulation.

Secondly, the  $X^{\text{tfm}}Y$  has previously been found, in development, to have normal levels of circulating T, and even higher than normal levels in adulthood [19, 20]. Thus, if aromatization is occurring normally within hippocampus, at either point in development, then it seems logical to us to assume that much of the  $X^{\text{tfm}}Y$  advantage over XX females shown here is due to the activity of estradiol following conversion from T. However, if this is the case, if estradiol is mediating the  $X^{\text{tfm}}Y$  advantage, then the diminished performance that the  $X^{\text{tfm}}Y$  males exhibit with respect to the wild-type male is likely due to some factor outside of the activity of the ER. Parsimony would suggest that this factor is the activity of the AR.

Further work will need to examine this hypothesis directly, quantifying the amount of aromatization within both the adult and developing hippocampus of the  $X^{\text{tfm}}Y$  animal, relative to XX and XY controls. Greater morphological analyses of the hippocampus also need to be carried out, to expand on this initial

data; there are a number of different potential mechanisms for the hypertrophy seen in the X<sup>tfm</sup>Y group, including increases in soma size and increases in cell number. Future investigations will also need to determine whether this result is due to changes in granule cells, interneurons, or glia.

In summary, we find that the *tfm*-affected male shows an intermediate pattern of performance on the water maze, seemingly female-like at the outset but reaching male-typical performance after a few days, suggesting that there is an AR-mediated component in this behaviour. The pattern of hippocampal morphology of the *tfm*-affected animals appears to be male-typical overall. An investigation into the estradiol content of discrete brain regions in male and female rat pups has shown similar estradiol levels in microdissections of the hippocampus immediately postpartum, with females showing higher levels in this structure within 32 h of birth [41]. Taken together, these lines of evidence beg a greater examination into the role of estradiol in the development of hippocampal morphology of normal animals, as well as the *tfm*; furthermore, this must be clarified in concert with a greater understanding of the function of the AR in the organization of this structure.

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Humans don't lordos. Humans don't mount. Our sexual and mating behaviors are quite various and overlapping between the sexes. And personal. So they don't get studied in the way rodent and primate sexual behavior are studied. They rely on self-reporting of sexual orientation and gender identification and non-invasive techniques. Sexuality is an aspect of self that feels so fundamental that individuals will undergo years of hormone treatment and surgery in order to modify their bodies to align with what they *feel*.

This section contains chapters that try to understand the neuronal basis of this aspect of self. They report on primate neuronal response during sexual behavior as well as human reports of gender identification and assignment. Selections in this section also explore the roots of dissatisfaction with phenotype and hence, the sex/gender one is assumed to be. Because human sexual identification is personal and deep-rooted, some of the experimental paradigms may seem a bit stilted and imposed. But ask yourself, How *does* one objectify something that is so taboo and difficult to understand objectively—from the outside?

Reading the chapters in this section will provide an understanding of the neuroscientific study of sexual identity. They are grouped together in such a way as to

1. Familiarize you with the spectrum of biologies that are compressed into two sexes;
2. Introduce the various ways in which sexual identity has been studied;
3. Elucidate assumptions used about what it means to be male or female in this scientific inquiry;
4. Make explicit the hypothesis underlying the interpretation of all of these studies: that organizational/activational mechanisms shape human sexual orientation and gender identification just as they shape rodent sexual behavior; and
5. Provide evidence that, just like vision and memory, sex is not unitary.

Finally, these studies raise the question of how to study the delicate interplay of experience and biology in shaping sexual orientation and gender identification.

### Female and Male

There seem to be strongly held assumptions about what it means to be a woman or to be a man. Humans make such pronouncements as “Women are from Venus and men are from Mars” or “Isn't that just like a (wo)man!” In the end, though, what elements of behavior underlie those statements? What neurons mediate those behaviors? This is what scientists whose work is understanding gender identification try to tease apart with varying success.

Slijper uses the condition of congenital adrenal hyperplasia (CAH) to gain some understanding on how androgens shape the XX brain. CAH girls have high circulating levels of androgens because their adrenals shunt the conversion of glucocorticoids into androgens. They also may have a serious salt imbalance that brings them into the hospital and under doctor's treatment often. Slijper reports that CAH girls have a higher level of tomboy behavior than other girls equally as sick. Her work supports the hypothesis that androgens shape the developing brain in a way that engenders “rough and tumble” play—even for humans.

The Mazur paper approaches the fundamental question of how strong a role nurture can play in the development of sexual identity, by following up on the outcome of sex assignment at birth of androgen insensitivity syndrome (AIS) babies. The failure of an earlier, very famous case of a male twin, David Reimer, who, because of a botched circumcision, was raised by his parents as a girl opened many questions about assuming that nurture was all in establishing gender identification. Here Mazur looks at the rate of reassignment as adults and finds that in his sample there are no documented cases for girls with complete AIS or boys and girls born with a micropenis and only a small percentage of reassignment for girls with partial AIS. Mazur concludes that sex of rearing is the most important factor in shaping the sexual identities of AIS females, supporting the notion that nurture is, indeed, critical in establishing gender identity. It is worth remembering, however, that AIS females do not have androgen receptors. This suggests that some

fundamental aspects of human male identity might be shaped by androgens qua androgens during development—much like the primate behavior of “rough and tumble” play, which is also reported in CAH females.

Deep and colleagues do the difficult work of correlating the variations of phenotype, genotype, and sex of rearing that emerge from treatment of infants with partial AIS. There are no recommendations other than one for further work toward the follow-up of different permutations and combinations of chromosomal sex, phenotypic sex, and gender of rearing. In sum, the paper convinces that simple alignment is not always the case, even within a syndrome.

Read these selections for their informed and compassionate attempts to understand the very difficult position in our society of being between the two ends of the spectrum. Ask yourself how much we have learned from these human variations on the themes of female and male. One can begin to imagine the interplay between biological factors and the strong cultural factors that try to tidy up what can be more ambiguous than we would like to believe: the tragedy of David Reimer and, yet, the success of the women in Mazur’s study caution us to look at each person individually. In light of the diversity of biologies they raise the question of whether, in an ideal world, we would continue to promulgate the ideals that male and female represent.

### Gay and Straight

An admission: I am a heterosexual female. I cannot claim first-person experience of gay sexual identification, or of really understanding gay male sexuality. However, it seems unlikely to me that the behavioral paradigms used in some of the papers in this chapter are analogous to human gay behavior. They seem more to rely on an assumption that gay male behavior is “feminized” behavior which in turn is interpreted as the gay brain being a feminized brain. Does human gay sexual behavior support this assumption? Regardless of whether it does or not, the papers in this chapter continue to convince us that like vision and memory, sex is not unitary—even heterosex.

Slimp and colleagues’ experiments demonstrate that, if, indeed, the medial preoptic area is involved in male sexuality, it is involved in the ability to have heterosex, not so much in the ability to desire heterosex. It is interesting to note that the Rhesus monkeys with MPOA lesions continue to masturbate and seek the help of the females to have erections, but they do not copulate. Although the authors interpret this as homosexual behavior, in our current climate that acknowledges a wide range of heterosexual behavior, it seems as if the monkeys may just need some Viagra.

The paper by Ehrhardt and her colleagues investigate the prenatal effects of estrogens on sexual orientation. They are extremely cautious in their interpretation of the finding that 25% of the women exposed to estrogens in development have homosexual or bisexual tendencies, but the suggestion is outstanding that hormones—and, specifically, estrogens—during development may play a role in establishing human sexual orientation. The assumption underlying this interpretation is that an estrogenized brain is a masculinized brain and a masculinized brain has an attraction to females.

Swaab and Hofman’s contribution is the first to report a structural difference between the brains of gay and straight males. Using the same methodologies as they used in their earlier work describing a sexually dimorphic nucleus in the human hypothalamus, they identify a difference in the size of the suprachiasmatic nucleus (SCN) between straight and gay males. When they compare the size of the nucleus across sex, they report that females and straight males have the same size SCN. Against the paradigm they conclude that a gay male brain is not a feminized brain.

Simon LeVay’s paper shows through simple and elegant anatomy a difference between gay and straight males in the structure of one of the hypothalamic nuclei described as dimorphic between females and males by Allen and Gorski, INAH 3—thus, supporting the interpretation of the gay male brain as feminized.

Allen and Gorski’s article takes us back to bilaterality as an indicator of a feminine brain. They report that gay males have anterior commissures that are larger than those of straight males and of the same size as those of females. This is a particularly interesting finding, because the anterior commissure is a structure identified with cognition and not sexuality and thus suggests that the gay male brain is shaped during development and not by sexual experience. The assumption remains, however, that the gay male brain is a feminized brain. Note, however, that in this study the brain of a self-described bisexual was analyzed with the brains of gay males. Note also that there are females with INAH 3’s as large as that of some straight males and straight males with INAH 3’s the same size as gay males.

Finally, the selection by Savic and colleagues addresses the issue of attraction by studying which smells are attractive to gay and straight males and straight females. Their findings, along with the assumptions of Allen and Gorski, and LeVay support that gay males are attracted to the same scent as are females which is the scent of other males—once again suggesting that the gay male brain is sexually comparable to the female brain.

Read these selections for the links they make with the original story of organization/activation by hormones early in development. Note that there are broad size ranges *within* groups and, as well, that it is very difficult to denote the boundaries of nuclei in the hypothalamus. The weight of the evidence contained here is that androgens may be more important for organization of the human sexual brain than it is for the rodent brain. Underlying all these interpretations is the assumption that the gay male brain is a feminized male brain. It is important to ask whether gay male sexual behavior supports this. Oddly, there is no work on gay female brains. Are the behaviors described by Ehrhardt and colleagues strong enough that one would expect to see structural differences in the brain? Where would you look? On the basis of what observation about female gay behavior would one expect their brains to be a masculinized or yet a third set of morphologies? Perhaps problematically for furthering understanding of sexualities all this work treats the categories “gay” and “straight” as self-evident.

### Transgendered and Gendered

Perhaps most intriguing and controversial is the search for the biological basis of the differences between individuals whose gender identification matches their phenotype and those who feel that their phenotype does not. As first person accounts relate, there is much turmoil involved in this misalignment—so much so that individuals will undergo long-term hormone replacement and genital surgery to make what feels wrong, feel right. The papers in this section chronicle the testing of various theories about the biological mechanisms at play in what is called, gender dysphoria.

Zuker and Green’s classic review of theories about gender dysphoria and how it develops supports the view that if not “born that way” these individuals certainly have dissatisfaction with their appointed genders early in life. The chapter provides insight into just how long term the distress and wish to realign are providing a strong case for the centrality of gender identification in one’s sense of self.

Gooren and colleagues reevaluate older evidence that male-to-female transsexuals have the surge of luteinizing hormone (LH) that marks endocrine patterns as XX. If this were true, it would be compelling evidence that male-to-female transgendered are born with feminized hypothalamic circuits much as those studied by Raisman and Field in the XX- and XY-androgen-deprived rodents. Gooren and colleagues find no evidence of an LH surge in male-to-females and therefore conclude that, at least in the domain of circuits controlling the secretion of reproductive hormones, male-to-female transgendered do not have feminized brains.

Zhou and colleagues demonstrate that the central region of the bed nucleus of the stria terminalis (BSTc), as stained with an antibody to vasointestinal protein (VIP) is significantly larger in males than in females and that the size of it in the XY female’s brain matches that in the XX female’s brain. It is also interesting to note that the size of the BSTc in the gay male brain is the same as in the straight male brain. Taken together, this evidence supports the hypothesis that the male-to-female transsexual’s brain is feminized while the gay male brain is not. Zhou and colleagues document the first difference between the brains of XX females, XY females and XY males.

Kruijver and colleagues follow up on this observation by reporting that the central subdivision of the bed nucleus of the stria terminalis of XY females resembles that of XX females in neuron number as well. Again, the number of neurons in the XY male is different.

Read these reports for how integral gender identification is to an individual’s sense of self, and the paradigm that shapes the design of the experiments that demonstrate a difference between XY females and XY males. Note that Swaab and his colleagues do not believe that the gay male brain is feminized but that the XY female’s is. Although this seems to match the behavioral observations a bit more closely, one still must be cautious about interpreting these data. Ask yourself how strong the evidence is for an organizational/activational mechanism underlying sexual differentiation in humans. If the evidence is not strong, should this paradigm be used to explain behavior? Again, as must be done for all the studies in this volume, one must ask what is driving the definition of gay male behavior as feminized. Do the papers in this section provide evidence as convincing as the evidence in the other sections? Note that the hypothesis of the male-to-female transsexual’s brain as somehow feminized during development and the female-to-male’s, as masculinized underlies the design and interpretation of these experiments as well. The logical extension of this view would then be that transsexuality has the same underlying causes as gay sexual orientation, an intellectual position that many transgendered individuals heatedly dispute. At this point in time, the evidence is provocative but very thin.



## Introduction

In the literature (Ehrhardt et al., 1968; Ehrhardt and Baker, 1974) the difference in gender role behaviour between congenital adrenal hyperplasia (CAH) girls and control girls is described as “tomboy” behaviour, by which is meant: expression of physical energy in outdoor games and sports; preference for playing with boys and boy’s toys rather than with girls and girl’s toys; little interest in marriage, pregnancy, motherhood and caring for young children; a preference for casual clothes (particularly trousers) and a minimal interest in jewelry, makeup and different hairstyles; the wish to be a boy rather than a girl. It is not made clear by the authors how many of these criteria are required in order to be called a “tomboy.”

The explanation for tomboy behaviour is sought by Ehrhardt et al. in the prenatal action of the male hormone. According to these authors, the male hormone has an “imprinting effect on the central nervous system,” which gives rise to tomboy behaviour. They find evidence for this hypothesis in rough-and-tumble play, which occurs more frequently in the male than in the female in both rhesus monkeys and small children. Young et al. (1965) found that female rhesus monkeys whose mother had received androgen injections in pregnancy exhibited rough-and-tumble play which resembled that of male control monkeys. In both CAH girls and CAH boys Ehrhardt and Baker (1974) found that the “vigorous muscular energy expenditure and intense interest in athletic sports” was greater than in brothers and sisters of CAH children.

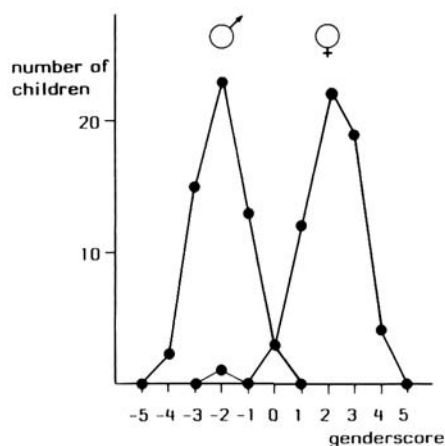
The question remains, to what extent are the results of animal experiments relevant for human behaviour? In the first place, tomboy behaviour in girls with excessive male hormone could, in principle, also be attributable to the way in which the parents experience the child’s deviant genitalia. CAH girls are born with genitalia resembling those of a boy. Experiments have shown that parents often exhibit significant different behaviour towards a baby who is dressed as a boy and has a boy’s name than towards the same baby dressed

as a girl and with a girl’s name (e.g. Will et al., 1976). Even immediately after delivery Out and Vierhout (1983) found that mothers exhibited significantly more emotional involvement (in handling, smiling, verbally responding to the baby) with a son than with a daughter. At any rate, the deviant genitalia can create doubts in the parents about the child’s sex. Secondly, CAH is a chronic sickness. The girls with the salt-loss variant of CAH especially—there are two forms of CAH, with and without salt loss—are frequently and often seriously ill during their first years of life. They experience frequent hospitalizations, checkups, etc. Moreover, these children have to undergo at least two genitalia operations (one immediately after birth and another in adulthood to correct the vagina). Furthermore, all of them have to take hydrocortisone throughout their lives. It is known of chronically sick children that they tend to react to a feeling of insufficiency with compensatory behaviour of a self-assured and bustling kind (Tavormina et al., 1976). One also frequently finds an anxiety for the future in these children which expresses itself in a low level of interest in marriage, motherhood and caring for small children (Schowalter, 1979).

## Observations on CAH Children

On the basis of the aforementioned problem definitions we decided to compare CAH girls with chronically ill girls in gender role behaviour. Diabetes mellitus as type of chronic illness seems to be the best match for CAH. The sample of CAH girls consisted of 10 girls with the salt-loss variant of CAH and 12 girls with the non-salt-loss variant. The total group of CAH girls ( $n = 25$ ) was compared with a group of healthy girls ( $n = 61$ ), a group of diabetic girls ( $n = 26$ ) and a group of sisters of CAH children ( $n = 10$ ). Similarly CAH boys ( $n = 19$ ) were compared with healthy boys ( $n = 56$ ), with boys suffering from diabetes ( $n = 22$ ) and with brothers of CAH children ( $n = 21$ ). The age range of all groups is from 7 to 17 years.

To measure the gender role behaviour of CAH girls an instrument was recently developed by myself—the



**Figure 74.1**

Distribution of frequencies of control boys ( $n = 56$ ) and girls ( $n = 61$ ) on the Sophia test.

Sophia test—which is based on the aspects of gender role behaviour distinguished by Ehrhardt et al. (Slijper, 1983). The values attached by the healthy children to outdoor play, indoor play, playing with boys, girls, dolls and cars, marriage etc. were measured. The children were asked to draw a person and to answer the question: “If I could have chosen between being born a girl or a boy I would have chosen. . . .” The Sophia test was given to a control group of healthy schoolchildren in primary and secondary education (ages 7–17 years).

The purpose of this study of the control group was to construct a scale on which boys were distinguished from girls. The principle for computing the gender score was based on the differences between the number of girls and the number of boys giving a particular response. A higher gender score means more “girlishness” (figure 74.1). The parents of sick children (CAH and diabetes) were interviewed using precoded questions about the children’s psychosexual and psychosocial development. Medical data were also collected, e.g. on the degree of virilization prior to the genitalia operation.

The study showed the following:

Sick girls (both CAH and diabetes) score significantly more towards the boyish side on the Sophia test (see table 74.1). Thus, the effect on gender role behaviour is not necessarily explained by hormonal action alone; *being sick plays a role*.

The CAH girls score significantly more to the boyish side than the diabetic girls (table 74.1); thus, there is also a specific CAH effect in girls. Closer examination of the data revealed that the *specific CAH effect is fully accounted for by the group of girls with the salt-loss variant of CAH* (table 74.2); with CAH girls with the non-salt-loss variant scoring about the same as diabetic girls (figure 74.2).

**Table 74.1**

Mean gender score, standard deviation and sample size for control children, children with diabetes, sick children (CAH + diabetes) and brothers/sisters of CAH children, by sex

	Boys			Girls		
	Mean Score	S.D.	<i>n</i>	Mean Score	S.D.	<i>n</i>
Control	−2.0	1.0	56	+2.2	1.1	61
CAH	−1.6	0.8	19	+1.0	1.2	25
Diabetes	−1.4	1.1	22	+1.7	1.0	26
Sick	−1.5	1.0	41	+1.4	1.1	41
Brother/sister CAH	−1.5	1.5	21	+2.2	1.0	10

**Table 74.2**

Mean gender scores of CAH children by sex and variant of CAH (salt-loss/non-salt-loss), sample size, standard deviation, and *P* value according to Student’s *t* test

Variant		<i>n</i>	Mean Score	S.D.	<i>P</i>
CAH ♀	Salt-loss	13	0.3	13.2	<0.001
	Non-salt-loss	11	0.9	5.0	
CAH ♂	Salt-loss	13	−1.4	10.0	N.S.
	Non-salt-loss	6	−1.6	4.2	

Analysis of variances (ANOVA) of the interview variables revealed that two groups of variables may be linked to the difference between salt-loss and non-salt-loss CAH girls: (a) a group of variables which indicate the seriousness of the CAH as a sickness, salt-loss CAH girls are more seriously ill than non-salt-loss CAH girls; (b) a group of variables which express parents’ doubts about the sex of the child, with doubts occurring in the case of salt-loss girls but not in the case of non-salt-loss girls.

In the group of girls with the salt-loss variant of CAH, those girls score most towards the boyish side who were originally registered as boys. These, however, are not the children who are most virilized; the objective degree of virilization does not correlate with the gender score.

CAH and diabetic boys score significantly more towards the girlish side on the Sophia test. Thus, being sick causes boys to score more towards the girlish side.

CAH girls do not differ from control girls in their appreciation of fighting, romping, wild play and outdoor play. However, more parents of a CAH daughter (80%) than of a diabetic daughter (50%) consider that their child is extremely fond of romping.

Diabetic girls differ from control girls in their appreciation of fighting (significantly more positive). CAH boys and diabetic boys differ from control boys in their appreciation of romping with father (significantly more negative). So chronically ill children seem to have

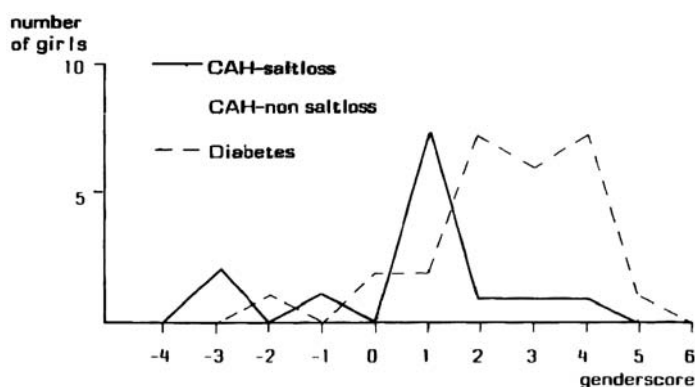


Figure 74.2

Frequency distribution of the gender score for CAH salt-loss ( $n = 13$ ), CAH non-salt-loss ( $n = 11$ ) and diabetic girls ( $n = 26$ ).

more problems with aggressive behaviour than healthy children.

Tomboy behaviour in CAH girls as described by Ehrhardt et al. was not found in the study. It was found, however, that CAH girls differed from diabetic and control girls with respect to the following: (i) appreciation of playing with cars (significantly more positive); (ii) expression of the wish to have been born a boy (significantly more frequent); and (iii) drawing of a man/boy as human figure (significantly more frequent). It is possible that parents' doubts about the sex of their child unconsciously (Daléry et al., 1982) generate doubt and uncertainty in the child about its true sex. This hypothesis points more in the direction of gender identity problems. The first requirement in dealing with these problems is therefore the prevention of doubt on the part of the parents about their child's sex.

### Concluding Remarks

Although it is impossible to separate the influence of androgen hormones from that of psychosocial factors on behaviour, most studies have not even considered environmental influences. Reinisch (1981), for example, found that females and males who were exposed during gestation to synthetic progestins showed a significantly higher potential for physical aggression than did their sex-matched unexposed siblings. However, the higher potential for physical aggression might also be attributed to the mother's fear of losing the baby (progestin is a treatment for imminent abortion). In our study, CAH boys and girls were not different from control children in their "appreciation" of fighting. Diabetic girls, however, showed a significantly more positive appreciation of fighting as compared to control girls. From our study, therefore, it seems more plausible that activation of aggression is related to sickness (e.g. physical interventions, hospitalizations etc.) rather than to prenatal hormonal factors.

A problem in comparing results of different studies is often the difference in method; Reinisch asked her children to react verbally on conflict situations, whereas we asked our children to give verbally their appreciation of fighting! In contrast to Reinisch (1981) Ehrhardt asserts that male sex hormone is not related to aggressive behaviour in the true sense but rather to rough-and-tumble play (Ehrhardt and Money, 1967; Ehrhardt et al., 1968; Ehrhardt and Baker, 1974). Indeed, CAH children and progestin-exposed girls exhibited more romping behaviour in our study than did control children of the same sex.

In our study there is no difference between CAH and normal girls as regards the appreciation of romping. However, more parents of CAH daughters than of diabetic daughters considered that their child is extremely fond of romping. Moreover, there is no difference in gender score between the group of CAH girls of which the parents consider their daughters fond of romping and the group of CAH girls of which the parents do not consider their daughters fond of romping.

The evaluation of the child's behaviour by parents is, therefore, not always the same as the evaluation of the behaviour by the child herself (Ehrhardt interviewed only the parents of CAH girls). As regards romping in boys: our study revealed an opposite result, with CAH and diabetic boys responding more negatively to romping behaviour than control boys. Again our study indicates a relation between romping behaviour and illness in general. Both Money et al. (1955), Money and Ehrhardt (1972) and Ehrhardt and Baker (1974) suppose that only the gender role behaviour of CAH girls is affected, not the gender identity. Our research suggests that there is uncertainty in CAH girls about their gender identity. The instability of the gender identity seems, in turn, to be related to the doubts of the parents about the child's sex.

In conclusion, the hypothesis that behaviour is masculinized by exposure to androgen hormones during

early stages of development cannot be supported by this study. Psychosocial factors such as the child being sick, and parents' doubts about the sex of the child seem to have more influence on gender role behaviour than does androgenic hormone action (i.e. degree of virilization).

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## Introduction

The traditional medical management of intersex infants has recently been challenged, especially in 46,XY infants assigned and reared female. The question of whether or not such gender assignment is appropriate and in the best interest of the child has been raised. To help answer this challenge, it is important to conduct long-term follow-up studies of such infants to ascertain whether or not they have established and maintained a gender identity commensurate with their initial gender assignment or show evidence of gender dysphoria and gender change. The purpose of this article is to review and summarize currently available data on gender outcome in 46,XY individuals with partial or complete androgen insensitivity or with the condition of micropenis.

Androgen insensitivity is an X-linked disorder of genetic male differentiation that is the result of an absent or defective androgen receptor (AR) gene. In this syndrome, the fetal testes function normally, but the utilization of androgens is impaired. The extreme variant of this condition is complete androgen insensitivity syndrome (CAIS). All 46,XY individuals with CAIS present with female-typical external genitalia. Internally, there are no Mullerian structures, such as a uterus or proximal vagina, due to the effects of the anti-Mullerian hormone secreted by the testes (along with testosterone and estradiol). In some cases, rudimentary Wolffian or male internal structures, epididymis and vas deferens, are present. Recently, residual activity of mutant AR-genes have been found to explain such internal male development. Hannema et al. (2004) suggest that individuals having any Wolffian development be classified as “severe” rather than as complete androgen insensitive. The distal vagina is usually short, dimple-like, with a blind ending. Infants with CAIS are always announced and reared as girls. The diagnosis is usually not made at birth. If the testes are not removed before puberty, their (normal) estradiol production will result in development of breasts. The testes must eventually be removed due to the risk of cancer and, after removal, life-long estrogen replace-

ment therapy is started in adolescence. Because of the lack of a uterus, CAIS individuals are amenorrheic, and the androgen insensitivity leads to sparse or absent body, axillary, and pubic hair.

If the genetic defect does not cause a complete block of the androgen receptor, a partial androgen insensitivity syndrome (PAIS) results, leading to variable hypoandrogenization and imperfect masculinization, despite normal androgen production. Presentation of the external genitalia is highly variable and can range from a penis with perineoscrotal hypospadias, with or without cryptorchidism, to a micropenis, with or without hypoplastic labioscrotal swellings, which may or may not be completely fused to form a scrotum. Remnants of female (Mullerian) internal reproductive structures have been found in some individuals diagnosed with PAIS. Infants with PAIS are assigned to either the male or female gender, depending to some extent on the degree of hypomasculinization. Virilization at puberty will also be variably incomplete.

The term micropenis denotes a completely differentiated penis with the urethral meatus at the tip and very small size. It is used as a clinical diagnostic term if the following criteria are met: (1) 46,XY karyotype, (2) testes, descended or undescended, (3) urethral meatus at the tip of the glans penis, i.e., no hypospadias, (4) stretched penile length (from pubic ramus to the tip of the glans) at or below 2.5 SD for age and stage of puberty (see table 75.1 in Lee et al., 1980). Lee et al. also describe a procedure to measure the penis and provide standards of penile length from 30 weeks of age through adulthood. Accordingly, in a 46,XY newborn, micropenis denotes a penis  $\leq 1.9$  cm stretched length; in the adult,  $\leq 9.3$  cm. 46,XY newborns with micropenis have been assigned to either the female or male gender, depending largely on the locally prevailing policy of medical management. Micropenis can occur in isolation or be associated with a variety of intersex syndromes.

The gender outcomes of principal interest in this article are (1) gender identity and (2) gender dysphoria. Gender identity refers to the basic sense of being a boy or girl, man or woman (Money & Ehrhardt, 1972).

Dysphoria denotes a state of dissatisfaction, anxiety, restlessness or fidgeting (Random House College Dictionary, 1988). For the purpose of this article, gender dysphoria is defined as a feeling of dissatisfaction or anxiety about oneself as male or female or dissatisfaction with one's assigned or legal gender.

## Method

For the purpose of this paper, I reviewed all articles on CAIS, PAIS, and micropenis cited by Zucker (1999), along with additional articles found via computer search of Medline, PsycINFO, and Cumulative Index to Nursing and Allied Health up to and including the year 2004. The search terms were male pseudohermaphroditism, androgen insensitivity syndromes, complete androgen insensitivity syndrome (CAIS), partial androgen insensitivity syndrome (PAIS), micropenis, intersex, ambiguous genitalia, microphallus, gender identity, gender dysphoria, and gender change.

Inclusion criteria for CAIS cases were the following: (1) identification of the AR gene mutation and/or a set of clinical criteria including (2) normal female-appearing external genitalia, (3) testes, (4) 46,XY karyotype, (5) no menses, (6) sparse or absent virilization, and (7) normal or high levels of testosterone. For PAIS, the ideal selection criterion would also be documentation of a defective androgen receptor gene, which constitutes the "gold standard" for the diagnosis (Migeon et al., 2002a). However, it is generally well known that it is difficult to demonstrate the presence of such a mutation in many cases. For instance, Migeon et al. (2002b) could demonstrate the presence of an AR-gene mutation in only 6 of 14 individuals classified as PAIS, despite abnormal AR-binding properties in cultured sex skin fibroblasts. For this reason, selection criteria for those with PAIS were either (1) presence of an AR-gene mutation and/or (2) endocrine findings and description of external genitalia that conventionally would permit the diagnosis of PAIS. The selection criteria for individuals with a micropenis are the clinical criteria defined earlier. The report by Reilly and Woodhouse (1989) was excluded because it did not meet these criteria. Only articles that clearly indicated the number of patients identified and their ages were utilized. The articles by Hinman (1972) and Slijper, Drop, Molenaar, and Keizer-Schrama (1998) were excluded because this criterion was not met. In addition, the case selection was based on information within the articles that allowed a reasonable judgment about the status of an individual's current gender identity and/or social gender and his/her history of self-initiated gender change. These criteria were not met by Money, Mazur, Abrams, and Norman (1981) where

all individuals were infants, and Dessouky (2003), who noted gender change from female to male in 14 individuals with a diagnosis of PAIS, but in abstract form only.

## Results

### Complete Androgen Insensitivity Syndrome

Table 75.1 summarizes the reports that met the inclusion criteria. They include a total of 156 individuals. The AR-gene mutation was found in 62 cases, all of which came from the most recently published studies (Hines, Ahmed, & Hughes, 2003; Hooper et al., 2004; Melo et al., 2003; Slijper, Frets, Boehmer, Drop, & Niermeijer, 2000; Wisniewski et al., 2000). None of these individuals initiated a gender reassignment to male. Most of the articles did not contain any information on gender dysphoria or atypical gender roles as defined by Zucker (1999, p. 7).

### Partial Androgen Insensitivity Syndrome

Table 75.2 summarizes data on 99 individuals diagnosed with PAIS, in whom 26 were found to have the AR-gene defect. Table 75.2 also shows that nine individuals with PAIS changed gender later in life of their own initiative. One was initially assigned male, but 5 days later the assignment was changed to female (Gooren & Cohen-Kettenis, 1991). This person reassigned herself to the male gender at age 30. The initial gender assignment of the second one was female, but was changed to male at 3 weeks (Minto, Liao, Woodhouse, Ransley, & Creighton, 2003). Masculinization was incomplete and, "although still legally a male" (p. 1255), the patient underwent feminizing genital surgery at age 30 and since then "lives as a woman" (p. 1255). The third person initiated reassignment from male to female at age 22 (Migeon et al., 2002b). Diamond and Watson (2004) reported the remaining six, two of whom changed from female to male and four changed from male to female. Two of these latter four were initially assigned as female but changed by a physician to male at ages 18 months and 3 months, respectively, when testes were palpated.

Gender dysphoria without complete gender change was also reported. Money and Ogunro (1974) reported on one individual who was reared as a "hermaphrodite girl" (p. 181). According to Money and Ogunro, this woman had thoughts or fantasies of "a woman haunted by phobic, obsessional doubt that other people, a sexual partner in particular, would easily divine her secret, namely that she was imperfect, an abnormal man/woman, so to speak" (p. 189). Interestingly, this woman had three siblings with the same condition (not available for study), two of whom were known to have changed to live as men.

**Table 75.1**  
Gender development in 46,XY individuals born with complete androgen insensitivity syndrome

General Information		Gender History						Latest Report		
		Androgen Receptor Detected	<i>n</i>	Neonatal Gender Assignment	Physician Imposed Gender Reassignment	Patient Initiated Gender Reassignment	Age at Patient Initiated Gender Reassignment	Age at Time of Study (Years)	Lives as (M, F, Other)	Gender Dysphoric (Yes, No, NI)
Reference	Country									
Morris and Mahesh (1963)	USA	NI	3	F	—	—	—	18, 18, 21	F	No
Teter and Boczkowski (1966)	Poland	NI	4	F	—	—	—	18–27	F	No
Masica, Money, and Ehrhardt (1971) <sup>a</sup>	USA	NI	10	F	—	—	—	17–34	F	No
Imperato-McGuinley, Picardo, Gautier, Voyer, and Bryden (1991)	Dominican Republic	NI	10	NI	—	—	—	18–76	F	No
Slob et al. (1993)	Netherlands	NI	4	F	—	—	—	19, 20, 49, 63	F	No
Costa et al. (1997)	Brazil	NI	2	F	—	—	—	14, 18	F	No
Slijper et al. (2000)	Netherlands									
Children		NI	15	F	—	—	—	NI	F	NI
Adults		10	10	F	—	—	—	24.11–70	F	No
Wisniewski et al. (2000)	USA	14	9 <sup>b</sup>	F	—	—	—	25–65	F	No
Hines et al. (2003)	UK									
Volunteers		NI	12	F	—	—	—	>15	F	No
Medical chart		10	10	F	—	—	—	>15	F	No
Melo et al. (2003)	Brazil		11							
Case 1		P904V		F	—	—	—	5.8	F	No
Case 2		S119X		F	—	—	—	9.5	F	No
Case 3		R779W		F	—	—	—	14	F	No
Case 4		R752X		F	—	—	—	14.5	F	No
Case 5		R855C		F	—	—	—	16	F	No
Case 6		M807V		F	—	—	—	16	F	No
Case 7		R855C		F	—	—	—	17	F	No
Case 8		N705S		F	—	—	—	19	F	No
Case 9		L768V		F	—	—	—	19.8	F	No
Case 10		N705S		F	—	—	—	34	F	No
Case 11		L768V		F	—	—	—	43	F	No
Hooper et al. (2004)	Brazil	17	17	F	—	—	—	5 were <18 12 were 18–74	F	No
Diamond and Watson (2004)	UK/USA	NI	39	F	—	—	—	NI	F	No
Total			156 <sup>c</sup>				0			0

*Note.* NI: no information.

<sup>a</sup>Same subjects as Money, Ehrhardt, and Masica (1968).

<sup>b</sup>Five subjects not counted, previously reported on in Masica et al. (1971).

<sup>c</sup>128 individuals 18 years and older, 28 individuals 0–18 years.

**Table 75.2**  
Gender development in 46,XY individuals born with partial androgen insensitivity syndrome

Gender History												
General Information			Age at				Latest Report		Gender			
Reference	Country	Androgen Receptor Detected	n	Neonatal Gender Assignment	Physician Imposed Gender Reassignment	Physician Imposed Gender Reassignment	Patient Initiated Gender Reassignment	Age at Patient Initiated Gender Reassignment	Time of Study (Years)	Lives as (M, F, Other)	Identifies as (M, F, Other)	Dysphoric (Yes, No, NI)
Morris and Mahesh (1963)	USA	NI	1	F	—	—	—	—	19	F	F	No
Teter and Boczkowski (1966)	Poland	NI	3	F	—	—	—	—	21, 21, 22	F	F	No
Money and Ogunro (1974)	USA	NI							13.5–39			
Group 1			2	F	—	—	—	—		F	F	Yes (1)
Group 2			8	M	—	—	—	—		M	M	No
Madden, Walsh, MacDonald, and Wilson (1975)	USA	NI	1	F	—	—	—	—	28	F	F	No
Assael, Lancet, and Shani (1976)	Israel	NI	1	F	—	—	—	—	40	F	F	No
Beheshti, Hardy, Churchill, and Daneman (1983)	Canada	NI							0.5–27			
Group 1			5	F	—	—	—	—		F	F	No
Group 2			12	M	—	—	—	—		M	M	No
Gooren and Cohen-Kettenis (1991)	Netherlands	1	1	F	—	—	M	30 years	33	M	M	No
Costa et al. (1997)	Brazil	NI	6	M	F	5 days	—	—	15–30	F	F	No
Migeon et al. (2002b)	USA	6	14									
Case 1				M	—	—	—	—	35–39	M	M	No
Case 2				M	—	—	—	—	20–24	M	M	No
Case 3				M	—	—	—	—	25–29	M	M	No
Case 4				M	—	—	—	—	45–49	M	M	No
Case 5				M	—	—	—	—	40–44	M	M	No
Case 6				—	—	—	—	—	35–39	F	F	No
Case 7				F	—	—	—	—	25–29	F	F	No
Case 8				—	—	—	—	—	30–34	F	F	No
Case 9				—	—	—	—	—	20–24	F	F	No
Case 10				F	—	—	—	—	20–24	F	F	No
Case 11				F	—	—	—	—	40–44	F	F	No
Case 12				F	—	—	—	—	35–39	F	F	No
Case 13				F	—	—	—	—	25–29	F	F	No
Case 14				M	M	—	F	22 years	45–49	F	F	No

	Melo et al. (2003)	Brazil	14		F	1 year	—	—	—	1	F	No
	Case 1	R855H		M	F	—	—	—	—	14	F	No
	Case 2	I898F		F	—	—	—	—	—	18	F	No
	Case 3	I898F		F	—	—	—	—	—	19	F	No
	Case 4	I898F		F	—	—	—	—	—	20	F	No
	Case 5	I898F		F	—	—	—	—	—	30	F	No
	Case 6	M742V		F	—	—	—	—	—	2.5	M	No
	Case 7	W741C		M	—	—	—	—	—	2.6	M	No
	Case 8	R855H		M	—	—	—	—	—	7.3	M	No
	Case 9	T602P		M	—	—	—	—	—	7.8	M	No
	Case 10	R840S		M	—	—	—	—	—	13.8	M	No
	Case 11	R855H		M	—	—	—	—	—	16	M	No
	Case 12	T602P		M	—	—	—	—	—	16.5	M	No
	Case 13	R840S		M	—	—	—	—	—	25	M	No
	Case 14	Y763C		M	—	—	—	—	—	18–70		No
	Minto et al. (2003)	UK	9	F	M	3 weeks	F	30 years	—		F	No
	Case 1	NI		F	—	—	—	—	—		F	No
	Case 2			F	—	—	—	—	—		F	No
	Case 3			F	—	—	—	—	—		F	No
	Case 4			F	—	—	—	—	—		F	No
	Case 5			F	—	—	—	—	—		F	No
	Case 6			F	—	—	—	—	—		F	No
	Case 7			F	—	—	—	—	—		F	No
	Case 8			F	—	—	—	—	—		F	No
	Case 9			F	—	—	—	—	—		F	No
	Mazur et al. (2004)	USA	3	M	F	.5 months	—	—	—	33	F	No
	Case 1	NI		M	—	—	—	—	—	33	F	No
	Case 2			M	F	18 months	—	—	—	31	F	No
	Case 3			M	F	1.5 months	—	—	—		F	No
	Diamond and Watson (2004)	UK/USA	19	M							F	No
	Case 1	5 <sup>a</sup>		M							F	No
	Case 2			M							F	No
	Case 3			M							M	No
	Case 4			F							F	No
	Case 5			F	M	6 years					M	No
	Case 6			F	M	5 years					M	No
	Case 7			F	M	1.5 years	F	34 years			F	No
	Case 8			F	M	2 weeks					M	No
	Case 9			F			M	18 years			M	No
	Case 10			F			M	30 years			M	No

Table 75.2  
(continued)

Gender History													
General Information				Physician Imposed						Latest Report			
Reference	Country	Androgen Receptor Detected	n	Neonatal Gender Assignment	Physician Imposed		Patient Initiated		Age at		Time of Study (Years)		Gender Dysphoric (Yes, No, NI)
					Gender Reassignment	Gender Reassignment	Gender Reassignment	Gender Reassignment	Physician Imposed	Patient Initiated	Age at	Lives as (M, F, Other)	
Case 11				F							F	F	No
Case 12				F							F	F	No
Case 13				F							F	F	No
Case 14				F							F	F	No
Case 15				F							F	F	No
Case 16				F							F	F	No
Case 17				F							F	F	No
Case 18				F							F	F	No
Case 19 <sup>b</sup>				F	M	3 months	F	22 years			F	F	No
Total			99 <sup>c</sup>				9						1

Note. NI: no information.  
<sup>a</sup> Authors refer to another article for the specific DNA conformation.  
<sup>b</sup> Additional case from p. 628 of article.  
<sup>c</sup> 81 individuals were 18 years and older, 17 individuals were 0–18 years.

### Micropenis

Table 75.3 summarizes the data on 89 individuals with a micropenis, 10 of whom were assigned and reared female. None of these 89 individuals were reported to have changed gender, regardless of whether the original assignment was as a boy or as a girl. Wisniewski et al. (2001) reported that only one male out of 13 was dissatisfied with his gender of rearing and doubted his gender, i.e., showed gender dysphoria, although about half of these persons said they were dissatisfied with their genitals and one man refused to answer questions about his sexual functioning and body image. However, while all five individuals reared female were currently satisfied with their gender identity, four (80%) of them said that at some point they had questioned their gender of rearing. Those reared male ( $n = 13$ ) reported being more masculine than those reared female ( $n = 5$ ) and the reverse held true for self-rated femininity. In the most detailed case report available on a male with micropenis, Money (1984b) documented the history of gender change ideation for a period of time in this person's life who, however, never put his fantasies into action.

### Discussion

Nine summary statements are justified based upon the findings of this review: (1) Gender identity as female was established and maintained in all individuals diagnosed with CAIS. (2) The majority of individuals diagnosed as PAIS and all of those born with a micropenis maintained their initially assigned gender, whether male or female. (3) Gender change occurred only in PAIS and (4) both from male to female and from female to male. (5) A specific AR-gene mutation was not demonstrated in every individual classified as PAIS or CAIS. (6) AR-gene mutations were documented in PAIS individuals regardless of the gender they developed. (7) AR-gene mutations were also documented in PAIS individuals who changed gender as well as those who did not. (8) No one with CAIS was described as gender dysphoric, although some individuals reported dissatisfaction with various genital and non-genital aspects of their body. (9) One individual with the diagnosis of PAIS and two males with a micropenis clearly demonstrated gender ambiguity, but none initiated a gender change even though one individual with a micropenis had a period of transsexual fantasies, and the other directly expressed dissatisfaction with his gender of rearing.

Three questions posed at the outset of this review can now be answered. Do any individuals with a diagnosis of CAIS, PAIS, or presentation of a micropenis at birth change from their initial gender assignment? Self-initiated gender change is documented for persons

diagnosed with only PAIS, not individuals with CAIS or micropenis. The second question asked about frequency of gender change. It is rare even in individuals with PAIS to change gender. Those who do are not the majority of individuals with this diagnosis. Consequently, gender change is the exception to the main finding of this review: the best predictor of gender identity outcome in adulthood is the initial gender assignment. The third question asked whether or not gender dysphoria was present in individuals who did not change gender. Here, the answer is less clear. While it appears that gender dysphoria was present in some individuals, the degree to which it was present (i.e., dissatisfaction with one or two body parts versus many, or just expressed as a global gender dysphoria), as well as its intensity or strength, were not well defined nor systematically studied in the extant reports.

The studies reviewed herein have a number of limitations. (1) Accuracy of diagnosis is sometimes questionable, especially in those cases of PAIS where no AR-gene mutation is found because PAIS in its clinical presentation can closely mimic other intersex conditions. (2) Thorough description with measurements of the appearance of the neonate's genitalia are virtually lacking in all studies reviewed. (3) The use of support groups to select participants also raises a problem of diagnosis, as noted by Hines et al. (2003). Diamond and Watson's (2004) entire sample consisted of individuals from support groups and volunteers who "had confirming diagnosis of the CAIS or PAIS form of the condition" (p. 625). Diamond and Watson provided no medical or endocrinologic information. Accuracy of diagnosis is most important if one wants to make predictions concerning ultimate gender identity outcome that are based on specific syndromes defined by endocrine and/or genetic criteria. This, of course, assumes that accurate medical diagnosis predicts such ultimate outcome. This assumption has yet to be validated, with the possible exception of CAIS. Another limitation of support-group samples is selection bias because individuals self select to join a group and are therefore not representative of the total population under study. (4) Sample sizes were small. This especially applies to the subgroup of female-assigned individuals with micropenis ( $n = 10$ ). Furthermore, there were only six in this subgroup of individuals reared female who were 18 years and older. The oldest was 29 years old. The young ages of this very small group of females are problematic in establishing final adult gender identity outcome with a high degree of confidence because it is known that some intersex individuals and some non-intersex transsexuals change gender in mid-life or later. Thus, expansion of the micropenis database for those assigned and reared female and a longer follow-up period are vital to confirming or challenging

**Table 75.3**  
Gender development in 46,XY individual born with micropenis

Gender History														
General Information				Gender History					Latest Report					
Reference	Country	n	Neonatal Gender Assignment	Physician Imposed Gender Reassignment	Age at Physician Imposed Gender Reassignment	Patient Initiated Gender Reassignment	Age at Patient Initiated Gender Reassignment	Age at Time of Study (Years)	Lives as (M, F, Other)	Identifies as (M, F, Other)	Gender Dysphoric (Yes, No, NI)			
Money and Mazur (1977)	USA	1	M	—	—	—	—	9	M	M	No			
Burstein, Grumbach, and Kaplan (1979)	USA	14	M	—	—	—	—	1wk-11.1	M	M	NI			
Money (1984a)	USA	1	M	F	3 weeks	—	—	17	F	F	No			
Money (1984b)	USA	1	M	—	—	—	—	28	M	M	Yes			
Money and Norman (1988)	USA	4												
Case 1			M	F	6 weeks	—	—	18.0	F	F	NI			
Case 2			M	F	6 weeks	—	—	10.6	F	F	NI			
Case 3			M	F	21 days	—	—	11.2	F	F	NI			
Case 4			M	F	9 days	—	—	13.1	F	F	NI			
Bin-Abbas, Conte, Grumbach, and Kaplan (1999)	USA							18–27						
Group 1 0–2 years		4	M	—	—	—	—	4 months–2 at start of Testosterone Rx	M	M	No			
Group 2 6–13 years		4	M	—	—	—	—	6–13 at start of Testosterone Rx	M	M	No			
Wisniewski et al. <sup>e</sup> (2001)	USA													
Group 1 Case 1		13	M	—	—	—	—	21–54	M	M	Yes			
Case 2			M	—	—	—	—		M	M	No			
Case 3			M	—	—	—	—		M	M	No			
Case 4			M	—	—	—	—		M	M	No			
Case 5			M	—	—	—	—		M	M	No			
Case 6			M	—	—	—	—		M	M	No			
Case 7			M	—	—	—	—		M	M	No			
Case 8			M	—	—	—	—		M	M	No			
Case 9			M	—	—	—	—		M	M	No			

Case 10	M	—	—	—	—	M	No
Case 11	M	—	—	—	—	M	No
Case 12	M	—	—	—	—	M	No
Case 13	M	—	—	—	—	M	No
Group 2	5						
Case 1	M	F	1.3	—	23–29	F	No
Case 2	M	F	3.3	—	—	F	No
Case 3	M	F	1.10	—	—	F	No
Case 4	M	F	1.6	—	—	F	No
Case 5	M	F	1.6	—	—	F	No
Husmann (2004)	20	—	—	—	18–30	M	No
Lee and Houk (2004)	22	—	—	—	18–32	M	No
Total	89 <sup>b</sup>	—	—	—	—	—	2

Note. NI: no information.

<sup>a</sup>Four subjects in study were previously reported on in Money, Lehe, and Pierre-Jerome (1985).

<sup>b</sup>71 adults, 18 children.

the current evidence. (5) Sample follow-ups were often incomplete because of participant refusal or investigators' inability to locate people. (6) In some outcome studies, the investigators were the same individuals as those who provided treatment. A principle of clinical research is the separation of clinical service from outcome assessment. Participants may report findings to please the service provider, and the provider may minimize negative or adverse findings. (7) A final limitation is assessment methodology. Assessment of both gender identity and gender dysphoria in most studies was by unstructured self-reports of the individuals investigated. This appeared to be especially true when evaluating the presence or absence of gender dysphoria in those who did not change gender. Consequently, judgments about gender dysphoria were based on self-report and impressions of the investigators. An improvement in future studies would be incorporation of measures that appreciate the multi-dimensional nature of both gender identity (Egan & Perry, 2001) and gender dysphoria. Measures are presently in construction that appreciate the complex nature of these two constructs and demonstrate the psychometric properties of reliability and validity (Zucker, 2005).

The information presented in this review is relevant to parents and professionals who must make a decision as to initial gender assignment in infants diagnosed with CAIS, PAIS, or micropenis. The diagnosis of CAIS is usually not made in infancy, but in adolescence when the girl fails to menstruate. Gender assignment at birth is female and is made automatically based on the perfect female appearance of the external genitalia. However, on occasion the diagnosis of CAIS is made when a newborn presents with female external appearing genitalia and the parents and their physician are expecting a male because amniocentesis had indicated XY chromosomes. In this case, the decision as to initial gender assignment is made more deliberate. The evidence provided herein supports the prevailing policy that such an infant diagnosed with CAIS be assigned and reared female.

In regard to infants presenting with a micropenis, the data on gender outcomes in this review do not favor one or the other gender of assignment. Nevertheless, I recommend raising most infants with micropenis as males. This recommendation is made because (a) no major medical interventions (e.g., feminizing surgery and later creation of a vagina) are necessary; (b) lifelong hormone (testosterone) therapy starting in adolescence to induce a male puberty may not be necessary although this depends on the diagnosis associated with the micropenis (e.g., hypogonadotropic hypogonadism); and (c) preservation of

possible fertility. However, the recommendation for male rearing is provisional for this reason. The literature to date documents that a female gender identity apparently without gender dysphoria develops in XY individuals born with a micropenis and assigned female. If future investigations can replicate this finding and show for female-reared persons that their quality of life, including sexual functioning and eroticism, is better than the quality of life in those reared male, then the recommendation to assign as male needs to be revisited.

Deciding gender assignment for infants with the diagnosis of PAIS remains challenging. Evidence reviewed herein does not provide clear guidelines. Diamond and Sigmundson (1997) proposed to base the gender assignment of infants with PAIS on the degree of virilization of the external genitalia, which is presumed to be a marker of androgen imprinting in the brain. However, the status of masculinization of the external genitalia is, at best, a crude estimate of such prenatal androgenization (Sobel & Imperato-McGuinley, 2004). Review of 46,XX individuals born with congenital adrenal hyperplasia (CAH) and varying degrees of virilization of the external genitalia suggests that prenatal androgens do not usually interfere with the development of a female gender identity (Dessens, Slijper, & Drop, 2005). Furthermore, gender change, although infrequent, has not been shown to be correlated with a specific AR-gene defect. The reasons for self-initiated gender change in some individuals diagnosed with PAIS have yet to be elucidated.

In summary, most individuals with one of the intersex syndromes reviewed herein develop a gender identity commensurate with the gender they have been assigned in infancy. Gender dysphoria and gender change do occur, but at a low rate. Assignment of gender, while taking biologic criteria into account, is a social event. Longterm investigation integrating prenatal biological with postnatal biological and psychosocial variables, examples of which have been suggested by some investigators (Houk, Dayner, & Lee, 2004; Mazur, Sandberg, Perrin, Gallagher, & MacGillivray, 2004; Meyer-Bahlburg & Blizzard, 2004), may yield clues as to why some individuals with the same diagnosis changed gender and why the majority did not.

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Androgen insensitivity syndrome (AIS) arises from target tissue resistance to androgen action because of mutations in the androgen receptor gene. The molecular defect causes a range of abnormal male development from infertility with gynaecomastia to complete XY sex reversal with a female phenotype (CAIS) (1). The diagnosis of AIS is often inferred from clinical and biochemical findings: a 46 XY karyotype, absence of Mullerian-derived structures, normal testicular histology, and age-appropriate androgen production by the testis. Such cases comprise the most frequent cause of male undermasculinization (2). PAIS represents a wide variation in the degree of undermasculinization ranging from ambiguous genitalia to mild hypospadias (3, 4).

A grading scheme has been proposed by Quigley et al. to describe the degree of masculinization in AIS individuals (3). Grades 1–5 define a range from predominantly female appearance (e.g., mild clitoromegaly or some labial fusion), to individuals with principally a male phenotype (e.g., with micropenis, hypospadias, or cryptorchidism).

This heterogeneity in phenotypic expression of androgen insensitivity is partly explained by a variety of AR defects but individuals with identical mutations may display widely variable phenotypes both within and between affected families (5–8). It is evident that no precise relationship exists between genotype and phenotype in AIS, particularly in PAIS, suggesting that other factors are contributing to the degree of masculinization. How sex of rearing is influenced by this variance is not clear, and management of the XY intersex infant remains a complex issue for the family and professionals.

More than 500 mutations in the AR gene have now been documented in AIS (9). CAIS is invariably the result of mutations in the AR gene (10). In contrast, only a minority of patients who have features consistent with PAIS have an identifiable AR gene mutation (11). This raises the question of what is the underlying cause in cases that are AR mutation negative? This form of PAIS has features consistent with some defect in AR signalling. We have previously excluded abnormalities

in some of the coactivators required for AR action such as ARA70 and ARA24 (12, 13).

Development of the male foetal reproductive tract is dependent on adequate androgens produced in a critical time and threshold-dependent manner (14). It is possible that some variants of PAIS such as isolated hypospadias may occur if androgen production is not optimal during the first and early part of the second trimester. There is an association between hypospadias and low birth weight (15, 16). Androgen production is generally normal in isolated hypospadias when studies are performed in postnatal life (17, 18). It is possible that cases of PAIS with a normal AR gene may represent a separate entity, which has an influence on management. This study was designed to compare the phenotype between cases of PAIS who were either ARmt or ARwt and to identify criteria, which differentiate between the two groups. An external masculinization score, previously validated in normal infants, was used to obtain a quantitative measure of the degree of undermasculinization.

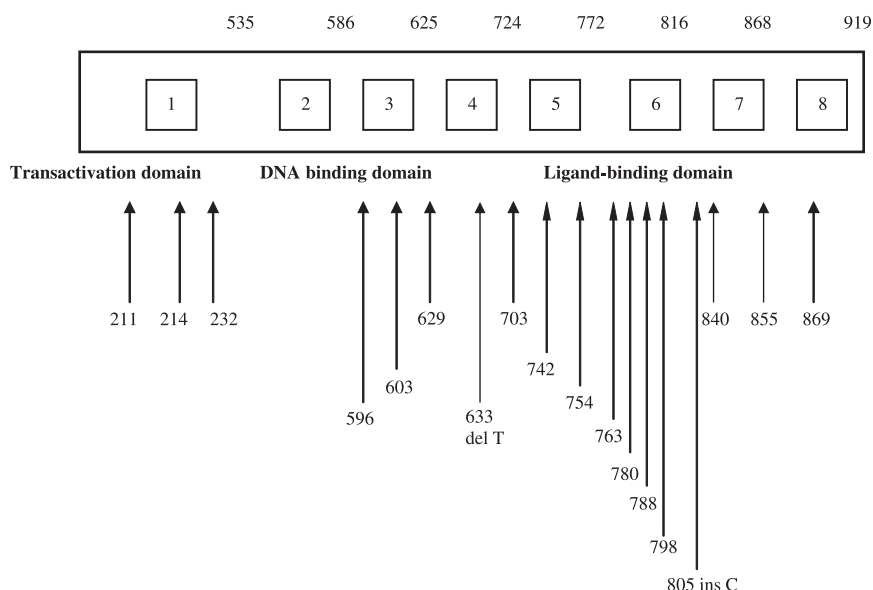
#### Subjects and Methods

Cases with a diagnosis of PAIS were identified from the Cambridge Intersex Database. For the purpose of this study, the following criteria were used to define a PAIS phenotype: severe perineoscrotal hypospadias with or without micropenis, bifid scrotum and undescended testes; an XY karyotype; normal or elevated basal testosterone for age; normal androgen response to hCG stimulation and normal testis histology, when available. An EMS was used to quantify the degree of undermasculinization (19). The parameters used to calculate the score are; presence of micropenis, scrotal fusion, site of urethral meatus and site of gonads. A maximum score of 12 represents normal masculinization. A family history of AIS and details of the sex of rearing were recorded.

If genital skin was obtained at the time of surgery, fibroblast lines were established and androgen binding was analysed as previously reported (20). For our laboratory, the range of binding capacity (Bmax) and

**Table 76.1**  
Details of genotype and phenotype in ARmt cases

Mutation	Cambridge PAIS Patients			McGill Database	
	EMS	kD (nM)	Sex of Rearing	Phenotype	Sex of Rearing
Exon 1	2	0.8	M	CAIS	F
Glu 211 Glu				PAIS	M
				PAIS	M
Exon 1	2	2.5	F	New mutation	N/A
Ser 232 Leu	2		F		
Exon 1	2	1.7	F	Normal	M
Gly 214 Arg	2	1.3	M	PAIS	M
	2.5	2	F		
Exon 3	6	1.9	M	PAIS	M
Ala 596 Thr				PAIS	M
				PAIS	M
Exon 3	2	5.5	F	New mutation	N/A
Arg 603 Lys					
Exon 4	3	0.9	M	New mutation	N/A
Arg 629 Trp					
Exon 4	3	Not done	F	New mutation	N/A
g.633 del T					
Exon 4	2	12.6	M	CAIS	F
Ser 703 Gly	3	Not done	F	PAIS	M
Exon 5	2	5.4	F	PAIS	F
Met 742 Ile					
Exon 5	1	3.5	M	PAIS	M
Phe 754 Leu				PAIS	M
Exon 5					
Phe 754 Ser	8	11.8	M	New mutation	N/A
Exon 6	8	2.2	M	PAIS	M
Tyr 763 Cys	2	3	M	PAIS	M
				PAIS	M
				PAIS	M
				PAIS	M
Exon 6	2	6.2	F	PAIS	F
Met 780 Ile					
Exon 6	8	3.3	M	PAIS	M
Arg 788 Ser					
Exon 6	3	1.4	F	PAIS	F
Gln 798 Glu					
Exon 6	5	Not done	M	New mutation	N/A
g.805 ins C					
Exon 7	11	2.9	M	PAIS	M
Arg 840 Cys	2		M	PAIS	M
				PAIS	F
Exon 7	1	3.5	M	PAIS	M
Arg 855 His	1	4.3	M	PAIS	M
				PAIS	M
				PAIS	F
				PAIS	F
				PAIS	F
				PAIS	F
				PAIS	F
				PAIS	F
Exon 8	5.5	3.1	M	PAIS	M
Ile 869 Met	6		M		

**Figure 76.1**

Location of mutations identified in the AR gene. The eight exons are shown. The amino acid residues are indicated in relation to the three principal functional domains of the AR. T and C refer to thymidine and cytosine, respectively.

binding affinity (kD) in normal genital skin fibroblasts is greater than  $300 \times 10^{-18}/\mu\text{g DNA}$  and  $0.8\text{--}1.7 \times 10^{-10} \text{ M}$ , respectively.

Mutational analysis of the AR gene was performed on genomic DNA extracted from peripheral blood. Exons 2–8 and nonpolymorphic regions of exon 1 were amplified by PCR and all products were sequenced directly in both directions using automated sequencing techniques as previously reported (20, 21). The amplified sequences were screened for mutations by direct sequencing.

When mutations were identified that were already recorded on the McGill International AR Database, the phenotypes and sex of rearing of the cases were compared.

### Statistics

A nonparametric test (Mann–Whitney) was applied to test the difference of variables between the two independent groups (ARwt and ARmt). A *P*-value of  $<0.05$  was considered a significant difference.

### Results

#### Number of PAIS Cases and AR Mutation Analysis

Two hundred and sixty-three cases with PAIS were identified, 111 of which had undergone AR sequencing at the time of the study. This group of cases formed the basis of the subsequent analyses. Nineteen mutations were identified in 27 patients (24%). Details of the phenotype, genotype, androgen binding affinity and sex of rearing in these cases are shown in table 76.1. The

mutations were distributed throughout the AR gene as shown in figure 76.1. Exon 1 contained three missense mutations, an unusually large number in this part of the gene causing PAIS; the majority of mutations were in exons encoding for the ligand-binding domain. The Gly214Arg mutation was found in three patients in this series.

All mutations were missense resulting in amino acid substitutions, apart from two: a deletion of tyrosine at codon 633 and a cystine insertion at codon 805. Reference to the McGill International Database indicated that six mutations identified in this study had not been reported previously. The majority of ARmt cases in whom androgen binding was studied had an abnormal binding affinity (kD).

### Comparison between ARmt and ARwt Cases

**Clinical and Biochemical Features** A positive family history was present in 61% of ARmt cases in comparison with only 21% in the ARwt group ( $P < 0.001$ ).

The median EMS was 3 in both groups; sex of rearing was male in 18 of 29 ARmt cases and 60 of 82 ARwt cases. The median EMS of 5 was again identical in both groups who were raised as male. Those raised as female had a median EMS of 2, whether ARmt or ARwt. There was a difference between the two groups for the EMS value above which a male sex of rearing was chosen. In the ARmt group, all with an EMS of 6 or more were raised male; for the ARwt group, this figure was 4 or more. For both groups combined, there was an equal ratio of male to

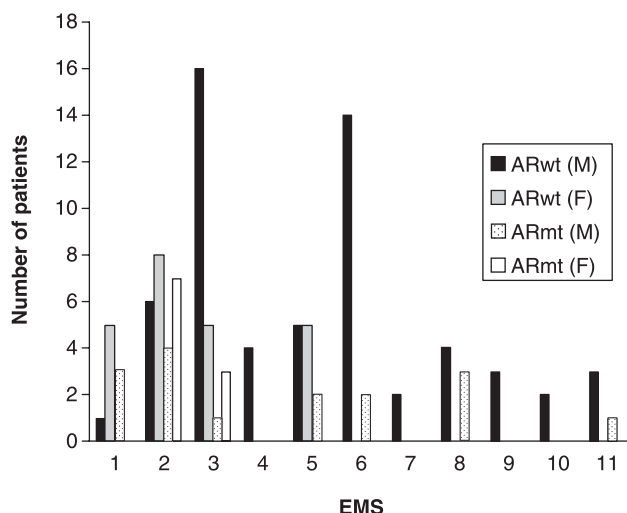


Figure 76.2

A comparison between the ARmt and ARwt cases in relation to the EMS and sex of rearing.

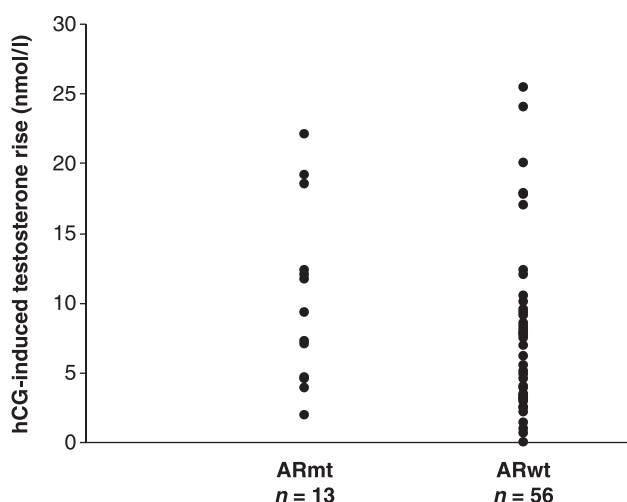


Figure 76.3

A scatter-plot showing the hCG-induced increment in testosterone secretion in ARmt and ARwt patients. The number of patients in each group is shown.

female sex of rearing for EMS values of 4 or less (figure 76.2).

An hCG stimulation test to assess androgen production in order to exclude an androgen biosynthetic defect was performed in 89 cases (13 ARmt, 56 ARwt). The median testosterone increment was 9.3 and 6.9 nmol/l in ARmt and ARwt cases, respectively; this difference was not statistically significant ( $P = 0.17$ ) (figure 76.3).

#### EMS and Specific Genotype

There was a concordance between the EMS in several cases, which had the same genotype. Thus, mutation

Table 76.2

Relationship between genotype, phenotype and sex of rearing

Mutation	Case No.	EMS	Sex of Rearing	Source
Arg840Cys	1	2	Male	Cam
	2 (sibling of 1)	2	Female	Cam
	3	11	Male	Cam
	4	2	Male	McGill
	5	6	Female	McGill
	6	3	Female	McGill
	7 (sibling of 6)	3	Male	McGill
Glu211Glu	1	2	Male	Cam
	2	0 (CAIS)	Female	McGill
	3	0 (CAIS)	Female	McGill
	4	NK	Male	McGill
	5	NK	Male	McGill
Gly214Arg	1	2	Male	Cam
	2	2	Female	Cam
	3	2.5	Male	Cam
	4	12 (normal male)	Male	McGill
	5	NK	Male	McGill
Ser703Gly	1	2	Male	Cam
	2	3	Female	Cam
	3	0 (CAIS)	Female	Cam
	4	2	Female	Cam

NK, details not known; Cam, Cambridge Intersex Database; McGill, McGill International Database.

Gly214Arg was found in three patients who displayed an EMS value of 2, 2 and 2.5, respectively. Similarly, two patients with the Ser703Gly mutation had an EMS value of 2 and 3, respectively. However, there were two patients in this series with an Arg840Cys mutation; both were raised male yet had EMS values of 2 and 11, respectively. Two mutations affecting the same codon (Phe754Leu and Phe754Ser) were associated with a highly variable EMS (1 and 8, respectively).

A comparison was made with the limited clinical details recorded in the McGill International Database for those cases, which shared the same mutation (table 76.2). In general, there was poor concordance between the genotype and phenotypes as defined by CAIS vs. PAIS, and sex of rearing. Two mutations (Glu211Glu, Ser703Gly) were associated with an EMS of 2 in two patients who were raised as male in this series of cases. These mutations had previously been reported in individuals with CAIS. Mutation Gly214Arg was associated with both male and female sex of rearing and has also been reported in a normal male (7).

#### Genotype, Phenotype and Sex of Rearing

Table 76.2 highlights four particular mutations associated with variable phenotypes and differences in sex of rearing. Seven patients with the Arg840Cys were identified from the Cambridge and McGill databases. The EMS in these patients ranged from 2 to 11; two sets of

siblings had the same EMS and yet had a different sex of rearing within the same family (sibling pair 1, 2 and sibling pair 6, 7). A further two patients with this mutation had an EMS of 2 and 11, respectively, and both were raised male.

Mutations Glu211Glu and Ser703Gly, which were associated with PAIS in our series, were reported as causing CAIS in three patients on the McGill Database. Mutation Gly214Arg is recorded on the McGill database in a normal male. In contrast, the mutation in our series was associated with an EMS of 2 in two patients (who were not raised the same sex) and an EMS of 2.5 in another patient.

## Discussion

The present study analysed a large number of cases in a well-defined PAIS cohort and is the first to use a validated, objective scoring system for assessing the degree of undermasculinization. This is important in view of the widely variable phenotype in patients with PAIS. Various parameters were assessed to define criteria that may differentiate ARmt from ARwt cases. However, we have demonstrated no significant differences between the two groups, apart from the association with a positive family history of AIS in the ARmt group. There was a tendency to a higher hCG-induced increment of testosterone in the ARmt group, but the result was not statistically significant. The observation needs to be validated using a uniform test protocol in a larger number of patients.

Does the PAIS ARwt group represent a distinct intersex disorder separate from some unknown forms of AR signalling defect? More than 70 AR interacting proteins have been shown to regulate AR function in vitro (See McGill International Database at [www.mcgill.ca/androgendb](http://www.mcgill.ca/androgendb) for full details). Co-regulatory factors are essential for optimal AR function and there is now evidence that some cases of AIS may be due to disruption of coactivator function (22, 23). Individual functional differences in AR coregulator proteins have been suggested to account for the phenotypic heterogeneity associated with identical mutations in the AR gene (24). We have previously excluded mutations in ARA70 and ARA24 (12, 13), but have found an association with a glutamine polymorphism in the SRC-3 coactivator in a group of patients with PAIS (25). However, routine screening for mutations or polymorphisms in this large family of proteins in patients with idiopathic undermasculinization is not justified.

Deficiency of 17 $\beta$ -hydroxysteroid dehydrogenase and 5 $\alpha$ -reductase are known defects in androgen biosynthesis and should be excluded in patients who have a PAIS phenotype (26, 27). P450 oxidoreductase (POR) is a protein that donates electrons to all microsomal

cytochrome P450 enzymes including 17 $\alpha$ -hydroxylase and 21hydroxylase (28). Consequently, POR deficiency shows biochemical features of combined 17 $\alpha$ -hydroxylase and 21hydroxylase enzyme deficiencies. Mutations in the POR gene have recently been reported (29). Affected girls/women are mildly virilized while affected boys/men have signs of undermasculinized external genitalia. It appears that a pathway of androgen biosynthesis, which uses androstenediol as substrate rather than testosterone to synthesize DHT may be operative only during foetal life in the human. This is based on maternal and foetal masculinization in affected girls/women being self-limiting, similar to that which occurs in placental aromatase deficiency (30). Such a pathway, termed "the backdoor pathway to dihydrotestosterone" is described in the tammar wallaby (31). It is possible that some cases of PAIS may be due to POR gene mutations and further studies are planned on our cohort of cases.

Identical missense AR mutations have been identified causing both CAIS and PAIS (5). Similar findings were observed in this series of cases in which the mutations Ser703Gly and Glu211Glu were shared between individuals with both PAIS and CAIS. In addition, we identified mutation Gly214Arg in three severely undermasculinized patients (two of whom were raised female) and yet this mutation has also been reported in a normal phenotypic male subject with oligospermia (7).

Identical amino acid changes have also been reported to be associated with varying degrees of PAIS in unrelated families, and variations in phenotype may even occur among siblings with the same mutation (5, 6). The phenomenon was further substantiated in this study of three patients with the Arg840Cys mutation. Two were siblings with an EMS of 2, in contrast to a third unrelated patient who had an EMS of 11. The two affected siblings had differed in their sex of rearing. A further four patients with the Arg840Cys mutation were reported on the McGill International Database. Their EMS varied between 2 and 6 (21, 32). Two siblings had the same EMS score but again differed in their sex of rearing. In our cohort, an EMS of 4 or less was associated with an equal split of a male and female sex of rearing. Even a marked difference in the EMS (2 and 11, respectively) observed in two patients with mutation Arg840Cys was associated with a uniform male sex of rearing.

This mutation has also been reported in large Chinese kindred in whom a highly divergent clinical phenotype was described (8). Fourteen male members were affected in this family. The EMS varied between 2 and 12; five members were only mildly affected to the extent that they fathered children normally. It is clear from these examples that there are other unidentified

factors that operate in foetal life to cause the intra and interfamilial variability of androgen resistance in AIS.

Little is known about the criteria that determine sex of rearing in infants with such diversity in the EMS. Many factors may influence the decision on sex of rearing in XY infants with various degrees of undermasculinization (33). The presence of micropenis may instigate a trial of testosterone treatment to assess penile growth before a final decision is taken on the sex of rearing. Some ARmt patients with PAIS do respond to high doses of androgens, which can be of therapeutic value (34–36). Local variation in surgical procedures and surgical expertise is undoubtedly an influencing factor, with changes in techniques constantly being recommended (37). Cultural factors are important determinants of the sex of rearing: In our experience, Asian families prefer an infant raised as a boy even if severely undermasculinized. We did not explore the reasons why a particular sex of rearing was chosen in this retrospective analysis of a large cohort of PAIS cases. A study of 14 cases of PAIS in Brazil showed that once sex was assigned at birth, this was maintained thereafter at puberty and appeared to be independent of phallus size (38). This is at variance with what happens at puberty in disorders of androgen biosynthesis such as 5 $\alpha$ -reductase deficiency where a re-assignment to a male sex is not unusual (39). There was also an equal ratio of male and female sex of rearing in the Brazilian series of PAIS cases but, in contrast to the present study, no quantitative assessment of the degree of undermasculinization was undertaken during infancy.

Our study of PAIS is the first to analyse phenotype and sex of rearing in relation to the appearance of the external genitalia, as defined by a validated, quantitative assessment. We have demonstrated a poor correlation between phenotype and genotype in a large series of PAIS patients recorded in two AR mutation databases. In addition, we have shown that there are no specific criteria to differentiate between ARwt and ARmt forms of PAIS, and a positive family history of AIS is an important clue to the presence of an AR mutation in patients with a PAIS phenotype.

There is a clear need for a large-scale prospective study of infants with both XY intersex and a PAIS phenotype. A co-ordinated strategy of assessment should include assessment of the external genitalia using a method such as the EMS, the use of uniform protocols of endocrine tests, mutational analysis of relevant genes and information about surgical and medical interventions. Above all, there is a need for outcome studies to document ongoing development of the external genitalia in the shorter term and sexual function in the longer term. Such studies will improve the understanding of the causes of XY intersex, allow

more reliable genetic counselling, and help families to make informed decisions about sex of rearing.

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## Introduction

Several neuroanatomical loci have been shown to be involved in the display of male sexual behavior. However, the single most critical region for the display of male sexual behavior appears to be the medial preoptic-anterior hypothalamic (MP-AH) continuum. This assumption is based upon several experimental approaches in rats and an extension of some of the basic findings in rodents to carnivores. Electrical stimulation of this brain area has been found to moderately facilitate mating activity in male rats in general and to dramatically increase mating activity in a few individuals (24, 41, 42). Stimulation of other forebrain areas, especially along the path of the medial forebrain bundle, has facilitated sexual activity in some instances, but there is no report of the dramatic increase as reported by MP-AH stimulation (2, 3, 8, 23). In several non-primates, lesions of the MP-AH area have been shown to eliminate completely or markedly reduce male copulatory behavior. This is true of the rat (9, 10, 15, 40), cat (14) and dog (13). Typically the most severely affected males showed little mounting behavior and no intromissions or ejaculations.

Work on the neural correlates of sexual behavior of primates is quite limited and none of the studies have specifically investigated the effects of bilateral MP-AH lesions on sexual behavior. Lesion studies on non-human primates have been limited to an examination of the effect of removal of the temporal lobes (19, 39) and amygdala (17) where such lesions have been found to increase sexual activity. In restrained monkeys, electrical stimulation of several regions of the brain has revealed that the MP-AH locus is one of the most effective sites for evoking penile erection (21, 22, 37) or ejaculation (36).

In freely moving rhesus monkeys mounting of ovariectomized (non-estrogen treated) females accompanied by intromissions and thrusting could be induced by electrical stimulation of a few sites in the dorsolateral preoptic area, dorsal portion of the lateral hypothalamus and ventral portion of the dorsomedial nucleus of the hypothalamus (30, 31). Stimulation of

the rostral putamen evoked penile erection in the restrained monkey, but in the freely moving monkey mounting, intromissions and thrusting were evoked by electrical stimulation only in the presence of an estrogen-primed female (32).

Interestingly, stimulation of the medial preoptic and some hypothalamic areas including dorsal, lateral and posterior regions, produced penile erection in the restrained monkey, but did not induce mounting in the freely moving animal (32).

Of the work in primates some clinical studies on human male patients relates most closely to the non-primate studies on the effects of MP-AH lesions. In a series of patients convicted of sexual offenses including “pedophilic homosexuality” and “violent hypersexuality” unilateral lesions were placed in areas extending from the ventro-medial nucleus of the hypothalamus rostrally to the medial preoptic area (7, 29, 38). Patients receiving these lesions were reported to have experienced a lessening of the tendency towards abnormal behavior as well as a reduction in sexual interest. In earlier work on human patients, Meyers (26, 27) performed bilateral lesions of the ansa lenticularis to relieve abnormal motor signs but found that his patients suffered complete loss of sexual interest and even inability to achieve erection. The procedure used by Meyers was believed by him to have possibly damaged the anterior hypothalamus and medial forebrain bundle which carries nerve fibers to and from the MP-AH area.

The involvement of the MP-AH region in the display of male sexual behavior in a variety of mammalian species is well established. However, little attention has been given to its involvement in various types of sociosexual behavior other than heterosexual copulation. For example, lesions of the amygdala, which reportedly increase sexual activity in monkeys, also lead to disturbances of social behavior involving withdrawal from social interactions, alteration in dominance relationships, and increased social fear (18). Thus, MP-AH lesions could have an effect on social interactions which in turn could be altering sexual behavior.

In dogs, MP-AH lesions that alter male sexual behavior do not affect dominance relationships but do markedly reduce sexually dimorphic urine marking behavior (13). Thus one might propose that the MP-AH region is involved in the mediation of sexually dimorphic behaviors other than sexual responses.

Another consideration is that previous studies dealing with MP-AH lesions have yielded little information regarding the question of the effects on the animal's capability to achieve erection and ejaculation in a context other than copulation with a female partner. Although in dogs MP-AH lesions did not block erection and ejaculation, as elicited manually by the experimenter (13), study of a species in which masturbation typically occurs would provide a direct answer to this question.

The rhesus monkey is an ideal animal in which to pursue these considerations. There is a great deal of descriptive and analytical data available dealing with sexual behavior. The copulatory pattern of the adult rhesus male includes several elements such as manual contact (placing two hands on the hips of the partner), mount with or without pelvic thrusting, erection, intromission and ejaculation, which can be scored independently (1). In addition to various aspects of copulatory behavior, yawning is a sexually dimorphic behavior which frequently occurs in mating tests. Finally, since masturbation is a normal part of a rhesus monkey's behavioral repertoire both in the laboratory and in the natural environment (5, 34), possible changes in this behavior can be observed along with an analysis of changes in heterosexual copulation.

## Methods

### Subjects

Eleven adult feral males, 8–12 years old, were selected from a group of 14 on the basis of achieving 40% or more ejaculations on at least 5 tests with gonadally intact, periovulatory females. These subjects had been in the laboratory 1.5–6.5 years. Males of fairly equal sexual performance, as judged by ejaculation frequency, were distributed between a group to be lesioned ( $n = 6$ ) and a group to be subjected to a sham operation ( $n = 5$ ).

Both ovariectomized and gonadally intact adult females served as stimulus partners. Eleven ovariectomized females were administered estrogens either by daily injections of 10–20  $\mu$ g estradiol benzoate for 9–12 days or subcutaneous implantation of Silastic tubing (4 cm long  $\times$  4 mm o.d.) filled with either estradiol benzoate (EB) or crystalline estradiol. The Silastic implants were replaced at 3 month intervals. Two ovariectomized females were left untreated. Females with intact ovaries were selected as test partners from a group of 31 females when they were near the time of

ovulation, presumably their period of greatest receptivity (6).

### Housing

All animals were housed individually in 2.5 cm wire mesh cages, 75  $\times$  75  $\times$  75 cm. The males were housed in one room which contained only adult males. The females were housed in colony rooms containing both females and males. The animal rooms were lighted from 06:00 to 18:00. All animals were fed monkey chow supplemented by fruit. Water was continuously available. Blood for a separate study on testosterone and luteinizing hormone levels was taken from all subjects at frequent intervals.

### Surgical Procedure

Lesions were performed stereotaxically by a modification (13) of the Hume and Ganong (16) technique using X-ray ventriculography. A custom constructed attachment was made for the Kopf 1700 stereotaxic instrument allowing radiographs of the basal forebrain to be taken in the lateral and dorsoventral planes.

Anesthetization involved induction with ketamine and maintenance of surgical anesthesia with fluothane (Halothane). X-rays were taken before the surgery began to determine the placement of a 3-inch, 22 gauge needle for the injection of a contrast medium into the lateral ventricles. Immediately after injection of 0.1 ml of the contrast medium (meglumine iothalamate (Conray)) lateral X-rays were taken to outline the third ventricle for visualization of related structures such as the massa intermedia of the thalamus, anterior commissure and optic chiasm. Oblique dorsoventral X-rays were utilized to determine the exact midline of the brain as indicated by the ventral part of the third ventricle. Taking into account X-ray magnification factors, exact coordinates were determined for placement of the lesioning electrodes. After withdrawing the injection needle and placing the electrode in the brain, another set of X-rays in both lateral and dorsoventral planes were taken. Since the contrast medium had dissipated by this time, and the position of the electrode relative to the third ventricle could not be visualized, a tracing of the ventriculogram, in which the third ventricle was outlined, was overlaid on the X-ray with the electrode placement to verify accuracy of electrode placement. If necessary, minor adjustments of the electrode were made. The electrode was placed bilaterally, one side at a time, using the above procedure for each side.

Lesions were made by the radiofrequency lesion technique using the Kopf Model K13882 thermistor electrode (tip diameter of 0.7 mm; tip exposure of 1.5 mm) and the Kopf Model K13883 radiofrequency generator. The current was raised gradually over 30 sec to an electrode tip temperature of 71–73 °C and main-

tained at that temperature for 1 min. Preliminary work had established that a temperature of 72 °C would make a lesion of 2–3 mm in diameter in the rhesus monkey basal forebrain.

Sham-operated control males were subjected to the same surgical procedure except that current was not passed through the lesioning electrode.

### Behavioral Testing Procedures

All tests for sexual behaviors were carried out with oppositely sexed pairs. Some tests were conducted in a test chamber 120 × 150 × 75 cm with a plexiglass front, wire mesh floor, and a white “Marlite” back. The female was placed in the test cage first. The entry of the male began the test. Two observers, sitting 4.0 m from the cage recorded the behavior on a standardized scoresheet. Aspects of sexual behavior recorded were: contacts, mounts, intromissions, intromissive thrusts, erection and ejaculation. Also recorded were presents by the male to contact by the female, yawns, threats, aggressive attacks and grooming. The following temporal parameters were recorded to the nearest 15 sec: intromission latency, ejaculatory latency, post-ejaculatory interval and grooming duration.

Tests were ended either 2 min following an ejaculation or after 20 min had elapsed without an intromission. If two or more mounts occurred during the last 5 min of a 20-min interval without intromission, the test was extended an extra 10 min.

Both “single-series” and “two-series” tests were given. A single-series test ended after the first ejaculation or a 20-min interval without intromission. A two-series test ended after the second ejaculation or after the first 20-min interval without an intromission.

Some tests of sexual behavior were conducted in conjunction with a lever pressing procedure which was used to assess the male’s willingness to perform a task to gain physical access to a female. Lever pressing tests were done in a 95 × 150 × 75 cm chamber divided into two equally sized compartments by a vertical, moveable plexiglass partition. The partition was raised electronically when the subject completed 10 lever presses. If the males completed the 10 lever presses within 20 min, a sexual behavior test was initiated immediately thereafter in the same test chamber. If the lever pressing schedule was not completed in 20 min, the male and female were returned to their home cages for at least 1 h, and then brought together for a single-series or two-series test of sexual behavior. Subjects were trained to lever press by shaping with food reinforcement.

### Behavioral Testing Schedule

Preoperatively all tests were conducted with 3 ovariectomized females implanted with Silastic capsules of EB

and 2 ovariectomized untreated females. Each male was tested 4 times with each female in two-series tests separated by at least 4 days. After the 20 tests were completed, each male was trained to lever press and then given 2 single-series tests with each of the 5 females in the lever pressing procedure.

Postoperatively, both single-series and two-series tests were administered with 3 different sets of stimulus females for a total of 38–48 tests. Beginning 7–10 days postoperation, the males were paired with the set of 3 ovariectomized, EB-treated females used in preoperative testing. Each male was tested with these females a total of 4–7 times during the first month and again from the fourth through the sixth months. All of the tests with this set of females utilized the lever pressing procedure. During the second through the eighth postoperative months, 10–14 tests were conducted with females with intact ovaries during a period of sexual receptivity. The first 4 of these tests for each subject used the lever pressing procedure, and the remaining 6–10 tests were standard, single series tests. Females in natural estrus were again used for 5 tests during the fourteenth postoperative month. A third set of 8 females was employed during the fourth through the eighth postoperative months. These females were ovariectomized and treated with estradiol. Each male was tested from 1 to 3 times with each of these females for a total of 15 tests per male. Of all postoperative tests, the sham-lesioned group received 22 two-series tests and the lesioned group received 24 two-series tests.

Two lever pressing tests with each male were also conducted with the 2 untreated ovariectomized females. In addition each MP-AH lesioned male was paired with an ovariectomized estradiol-treated female in the male’s home cage and observed by time-lapse video-tape for 10 h.

### Observations of Masturbation and Seminal Expulsion

Ejaculation in the home cage was inferred from the presence of a clump or string of seminal material in the drop pans beneath the cages. Preoperatively, 70 days of observations were made over a 4.5 month period. Postoperatively, 120 days of observations were made during the first 8 months. During the fifteenth postoperative month, 30 days of observations were made.

Direct observation of masturbation by the male in his home cage was achieved with time-lapse video-tape recording. This was done only during the postoperative period and consisted of monitoring 10 h during the day at 7 months postoperatively for each lesioned male. Only the presence or absence of masturbatory movements were noted. At 15 months postoperation, both sham- and MP-AH-lesioned males were taped for 10 h on 2 different days, one week apart. Masturbation was

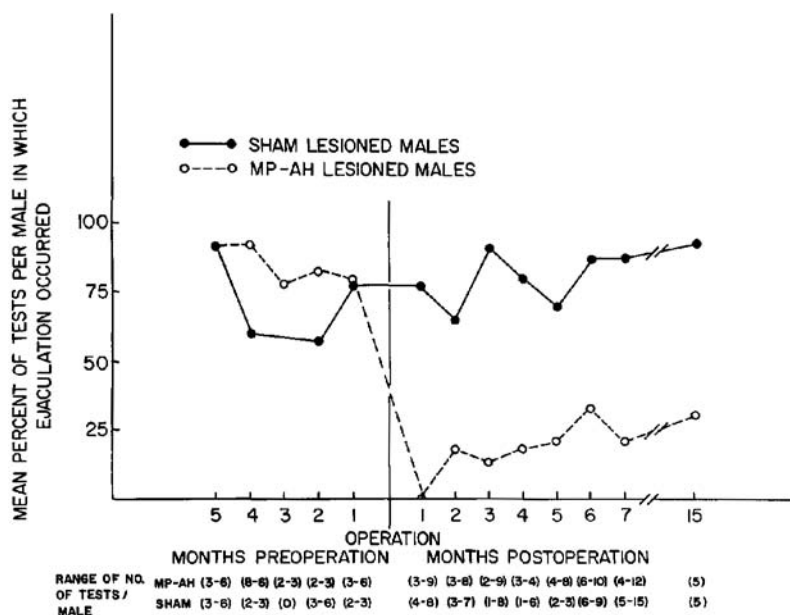


Figure 77.1

Percentage of tests per month in which ejaculation occurred for MP-AH and sham-lesioned subjects.

recorded only if there was clearly observable manipulation of the genitalia by the hands. Any instance of masturbatory movements was recorded as a bout and a session was defined as a collection of bouts not separated by more than 20 min.

#### Histological Technique

At 16 months postoperation, all lesioned adult males were anesthetized and perfused with saline followed by 10% formalin (4). Two to 3 h after perfusion the cerebral cortex was exposed and the head placed in the stereotaxic instrument. Coronal cuts were made with a scalpel attached to the electrode holder 10 mm anterior and 15 mm posterior to the plane of the lesion. Alternate sections were cut at 50  $\mu$ m and stained with cresyl violet or the Weil stain (for myelin) or left unstained.

Analysis of the lesions involved computation of lesion coordinates with respect to the anterior commissure, volume, and description of major areas destroyed.

#### Results

All sham and lesioned subjects recovered from the surgery rapidly and within 4 days appeared normal in their home cages.

#### Heterosexual Copulatory Behavior

In preoperative testing all 11 subjects copulated with ovariectomized, EB-treated, female partners; 9 of the 11 males ejaculated with all 3 females and the remaining two males ejaculated with 2 of the 3 females. The sham and lesioned subjects ejaculated once in a mean of 76.2% and 85% of preoperative tests, respectively.

Tests on which 2 ejaculations occurred were recorded on a mean of 55% and 43% of all preoperative two-series tests for the sham and lesioned groups respectively, with 10 of the 11 subjects ejaculating twice on at least one of these tests. In 12 tests with uncreated ovariectomized females, none of the subjects ejaculated.

There were 16 months of postoperative testing and, during this period, each subject received from 38 to 48 tests. In postoperative testing there was no decline in any measure of heterosexual activity in the sham-lesioned subjects (figures 77.1 and 77.2).

None of the lesioned subjects ejaculated during the first postoperative month (figure 77.1). Throughout the total postoperative test period there was a pronounced reduction in percentage of tests in which contacts, mounts, intromissions, or ejaculations were observed, regardless of whether the female partner was in natural or induced estrus (figure 77.2). Two lesioned males failed to display intromissions or ejaculations during the entire 16-month postoperative period. One of these males (1158) never mounted and displayed contacts on only 10% of all tests. The other male (1154) mounted occasionally but this was mostly with intact sexually receptive females. Two lesioned males (1153 and 1629) showed a severe impairment of copulatory activity and ejaculated only with intact, receptive females, one doing so on 8 occasions and the other on 3 occasions. The first ejaculations postoperatively for these subjects were in the third and sixth months, respectively. These males mounted on 47% of all postoperative tests. Another male (1625) ejaculated on 4 occasions with intact receptive females and on 4 occasions with ovariectomized, EB-treated females (3 of

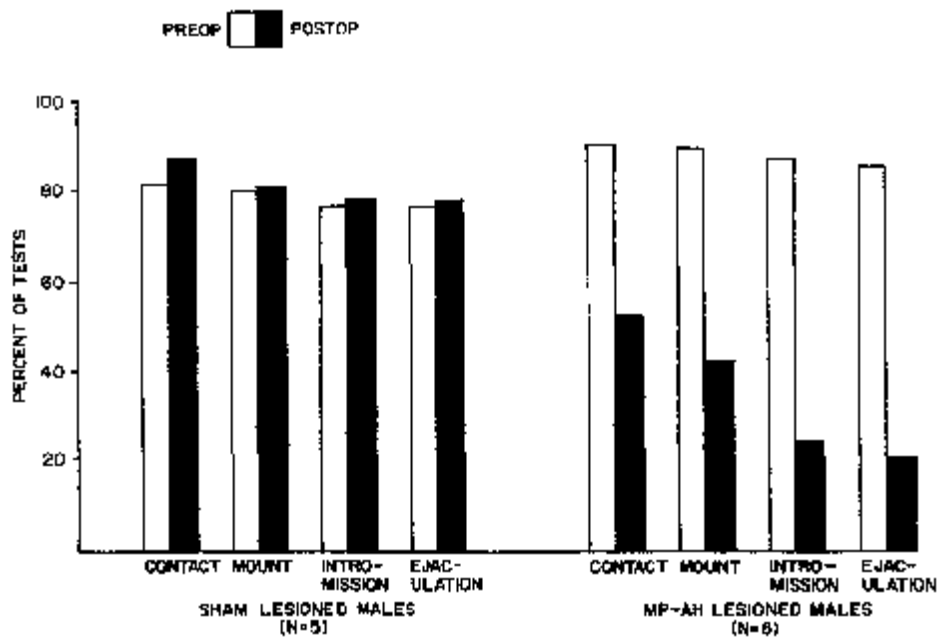


Figure 77.2

Percentage of all preoperative and postoperative tests in which contact, mount, intromission and ejaculation were displayed. The preoperative data is based on 18 tests and postoperative data on 38–48 tests per subject.

these with the same female) for a total of 8 ejaculations. The first ejaculation for this subject postoperatively occurred in the fifth month. Lesioned male 1624 first ejaculated during the fifth week postoperatively and thereafter ejaculated on 100% of the tests.

A comparison of preoperative to postoperative tests (table 77.1) revealed that when an ejaculation occurred there was an increase for all MP-AH lesioned males in the frequency of contacts, mounts and intromissions per ejaculation and in latency to intromission and ejaculation. A decrease in thrusts per intromission was observed in 3 of the 4 males that copulated postoperatively. There was no such trend for the sham-lesioned males.

In all postoperative two-series tests, the sham-lesioned subjects ejaculated twice. Similarly, the lesioned male with complete recovery ejaculated twice in all of his two-series tests. However, none of the other lesioned subjects ever ejaculated more than once in any two-series tests. When the 5 lesioned males with partial or complete impairment of copulatory activity were paired with females for 10 h in their home cages for observation by time-lapse video-tape recording, only one subject (1625) ejaculated. He failed to show additional intromissions in the 6 h of observation following the initial ejaculation.

#### Masturbation and Seminal Emission in the Home Cage

The appearance of seminal plugs in the drop pans revealed no overall decline from preoperative to postoperative levels in either sham or lesioned subjects

(figure 77.3). Both groups showed an immediate postoperative decrease in plug frequency, but by the end of the second month levels typical of the preoperative period were again observed. The seminal plugs found were believed to reflect the occurrence of regular bouts of masturbation, and direct visual observation and video-tape recording appeared to substantiate this. Prior to the employment of video-taping, lesioned male 1625 was observed to masturbate to ejaculation during a sexual behavior test in which no copulation took place and lesioned male 1158 and sham-lesioned male 1622 were observed to masturbate to ejaculation twice each in their home cages.

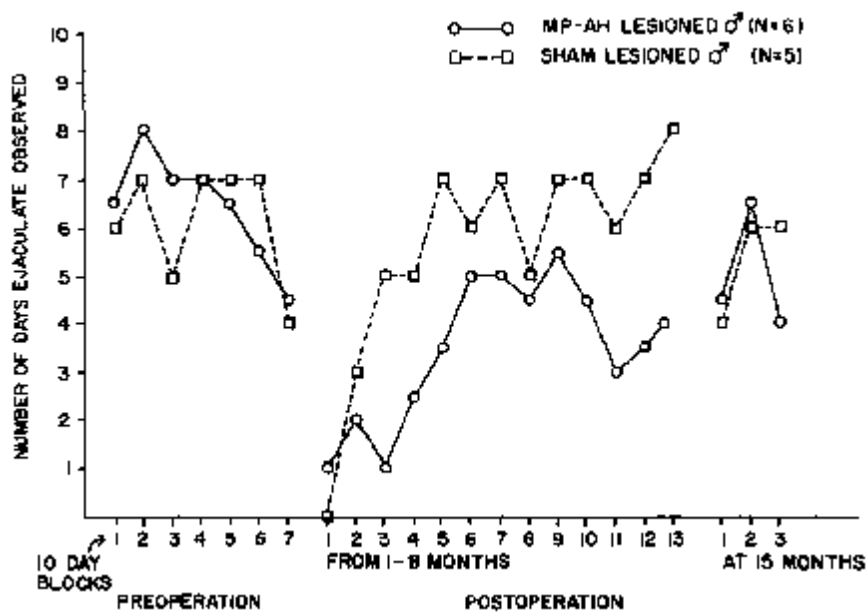
Seven months after surgery, time-lapse video-tape recording was employed to determine if the lesioned males which had not been previously seen masturbating were engaging in this activity. Analysis of tapes representing approximately 10 daylight hours verified that males 1154, 1629, 1153 and 1624 did masturbate. During the fifteenth postoperative month, each lesioned and sham-operated male was observed for two 10-h periods with video-tape. A masturbation bout was not scored unless the subject was seen stroking the penis. All males were seen to masturbate at least once to ejaculation. The lesioned males exhibited a mean of 9.33 ( $\pm 1.78$  S.E.) masturbation sessions with a mean of 3.72 ( $\pm 0.77$ ) bouts per session during the total 20 h. The sham-lesioned males displayed a mean of 6.00 ( $\pm 3.01$ ) masturbation sessions with 1.83 ( $\pm 0.60$ ) bouts per session in 20 h.

**Table 77.1**

Different components of sexual behavior of MP-AH and sham-lesioned subjects for all tests in which an ejaculation occurred

	No. of Tests	Mean Frequency of:				Mean Latency (sec)	
		Contacts	Mounts	Intromissions	Thrusts	Intromission Latency	Ejaculation Latency
<i>Sham Lesioned</i>							
Preop group mean ( $\pm$ S.E.)	10–18	3.65 (0.31)	3.51 (0.35)	2.76 (0.20)	8.78 (0.43)	2.51 (0.66)	1.53 (0.08)
Postop group mean ( $\pm$ S.E.)	23–45	4.23 (0.96)	3.96 (0.98)	2.77 (0.44)	9.15 (0.62)	3.39 (1.04)	1.68 (0.66)
<i>MP-AH Lesioned</i>							
Subjects							
1624							
Preop	18	1.72	1.11	1.11	8.86	3.60	0.23
Postop	41	4.04	3.88	2.45	10.53	4.37	1.59
1625							
Preop	18	2.47	2.16	1.83	9.98	3.37	0.73
Postop	8	6.87	6.62	4.62	6.74	7.09	2.06
1629							
Preop	15	3.20	3.40	2.46	7.75	4.36	1.15
Postop	3	16.66	16.33	5.66	5.14	13.08	9.16
1153							
Preop	13	5.07	4.69	1.69	11.03	4.38	0.82
Postop	8	12.12	11.62	2.25	9.51	10.46	2.93
1154							
Preop	11	7.18	7.00	1.72	11.60	7.11	2.20
Postop	0*	—	—	—	—	—	—
1158							
Preop	17	17.47	16.11	13.29	5.02	4.89	7.88
Postop	0*	—	—	—	—	—	—
Preop group mean ( $\pm$ S.E.)		3.11 (0.71)	2.84 (0.77)	1.52 (0.39)	9.40 (0.70)	3.92 (0.25)	0.73 (0.19)
Postop group mean ( $\pm$ S.E.)		9.92 (2.80)	9.61 (2.80)	3.74 (0.83)	7.98 (1.23)	8.75 (1.90)	3.93 (1.76)

\*These subjects displayed no ejaculations postoperatively.

**Figure 77.3**

Median number of days per 10-day block that ejaculation was observed in the home cage of MP-AH and sham-lesioned subjects.

### Lever Pressing Tests

During preoperative and postoperative testing there was no difference between the number of tests in which subjects lever pressed for access to ovariectomized, estradiol-treated females, ovariectomized untreated females, or intact females. During preoperative testing, subjects of the sham-lesioned group lever pressed for all females significantly more than those of the lesion group ( $\chi^2 = 7.61$ ,  $P < 0.01$ ); however, postoperatively this difference was not present, revealing that the lesioned males' performances actually improved in this regard.

### Social Behavior during Heterosexual Tests

In general the social interactions between the subjects and female partners throughout the experiment could be characterized as normal. No aggressive encounters were observed either pre- or postoperatively in sham or lesioned males. Threatening of the female, which was never observed preoperatively, occurred in only 4 of 226 postoperative tests for the sham-lesioned males and in 1 of 297 postoperative tests for the lesioned males. Grooming was especially prevalent postoperatively in lesioned subjects. These males received grooming for an overall mean of 19.3% ( $\pm 2.9$  S.E.) of the test durations. Preoperatively these subjects were groomed less than 5% of the time. Sham-lesioned males were groomed preoperatively and postoperatively less than 5% of the observed time.

Yawning behavior was not affected by MP-AH lesions. At least one yawn was recorded in a mean of 82.1% ( $\pm 6.0$ ) of the postoperative tests in lesioned males compared to 69.4% ( $\pm 11.1$ ) of the preoperative tests. In sham-lesioned males yawning was recorded in a mean of 63.6% ( $\pm 11.2$ ) of the postoperative tests and 46.7% ( $\pm 10.8$ ) of preoperative tests.

Preoperatively none of the males of the sham or lesioned groups presented to contacts by the female (the female placing her hands on the hips of the male). Postoperatively all 5 lesioned males in which copulatory behavior had been reduced or eliminated were contacted by females from 6–81 times and displayed presentations on 2–68 occasions per male. On many occasions females then mounted the males. The sham-lesioned adult males were contacted by females on only 3 occasions post-operatively and only 1 male presented on one of these occasions.

### Neurohistology

The borders of the MP-AH lesions were clearly distinguishable by the lack of neuronal elements, although the lesioned area was partially filled in with glial cells. Gross distortion of the third ventricle was not evident. No estimation of collapse of the surrounding neural tissue towards the lesioned area was made.

Figure 77.4 shows sections of each lesioned animal taken through the plane showing the largest extent of the lesion. A sagittal reconstruction, drawn from the frontal sections at 1.25 mm lateral to the third ventricle is shown in figure 77.5.

On the anteroposterior axis the lesions were located at the junction of the medial preoptic area and anterior hypothalamus. The center of the lesions varied from a plane passing through the posterior border of the anterior commissure to a position 0.45 mm anterior to this. The right and left lesions of males 1154 and 1158 were slightly asymmetrical (by about 0.30 mm) in the anteroposterior axis.

In the lateral plane, lesions extended 1.8–2.9 mm from the wall of the third ventricle. Lateral asymmetry did not correlate with the extent of sexual behavior deficit. For example, the two most asymmetrical lesions in the lateral plane were found in male 1629, who showed little recovery of sexual performance, and male 1624, who eventually displayed normal sexual behavior.

The greatest variability in lesion placement was in the dorsoventral plane (figure 77.5). The lesions of the 3 animals which showed the greatest deficit in sexual activity (1158, 1154 and 1629) were centered 0.30–1.4 mm below the ventral edge of the anterior commissure. The lesions of 3 subjects with the least impairment in copulatory performance were either dorsal (1624) or ventral (1625 and 1153) to those with the most severe impairment. In the latter two subjects, the lesions extended into the optic chiasm and partially damaged the suprachiasmatic nucleus.

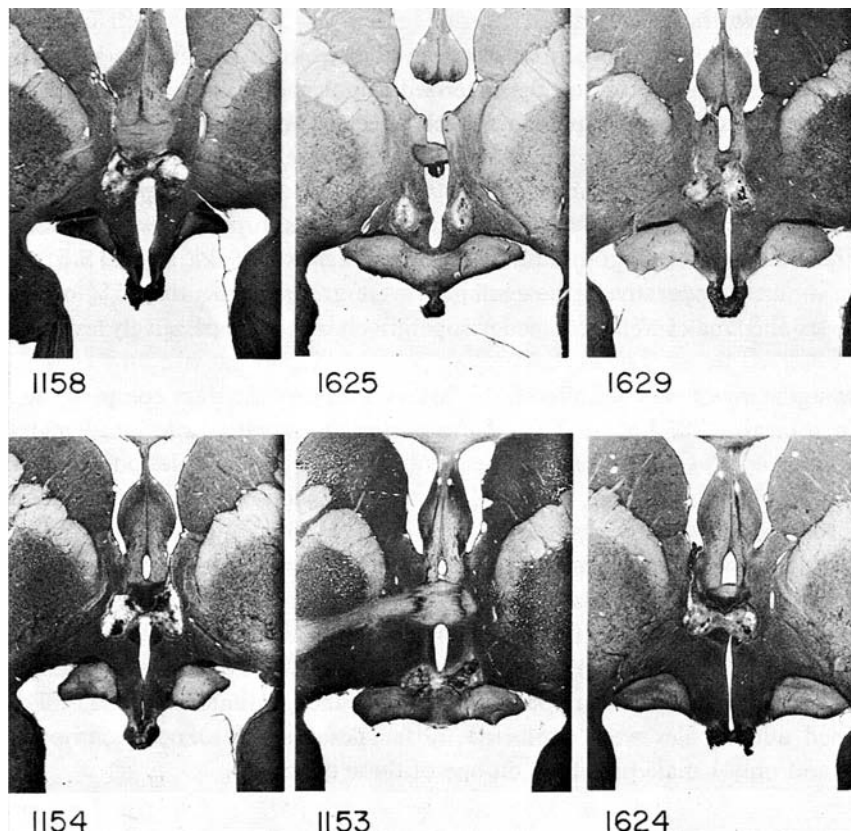
The total volume of tissue destroyed bilaterally was as follows: 13.3 mm<sup>3</sup> for male 1158, 12.4 mm<sup>3</sup> for male 1154, 12.5 mm<sup>3</sup> for male 1629, 11.3 mm<sup>3</sup> for male 1625, 11.6 mm<sup>3</sup> for male 1153 and 8.8 mm<sup>3</sup> for male 1624. The volume of the lesions correlated roughly with the extent of behavioral deficit in that the 3 males with the largest lesions had the greatest behavioral deficit, and the subject with the smallest lesion showed complete recovery.

### Discussion

The approach of the present study was to examine the role of the MP-AH area in sociosexual behavior through the observation of lesion effects on several behavioral categories. Heretofore, analysis of MP-AH lesions in male animals has been restricted mostly to heterosexual copulatory behavior.

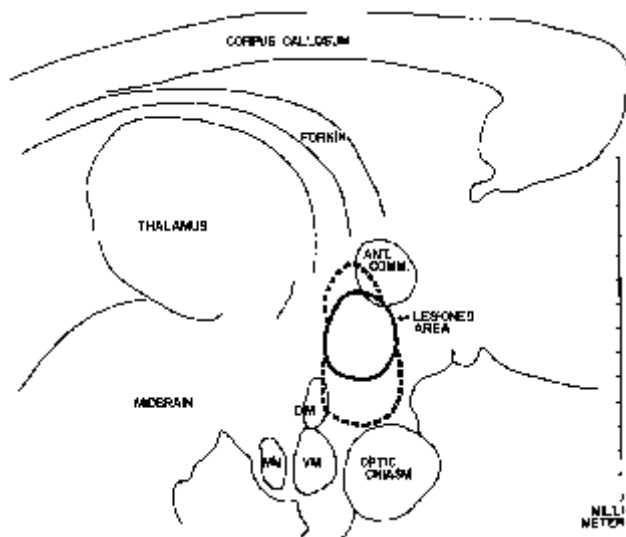
### Sexual Behavior

It could be proposed that copulatory behavior and masturbation are different behavioral expressions of male sexuality. The results of the present study indicate that lesions of the MP-AH region do not affect both



**Figure 77.4**

Photographs of frontal sections taken through the largest portion of the lesions of each male. Sections were cut 50  $\mu$ m thick and stained with cresyl violet.



**Figure 77.5**

Sagittal drawing approximately 1.5 mm lateral to the midline. The central area common to the 5 lesioned subjects with impairment of copulatory behavior is indicated by a solid line. The variation in the 3 subjects with the most severe impairment is indicated by the dotted line. Abbreviations: mm, mammillary bodies; VM, ventromedial nucleus; DM, dorsomedial nucleus; ant. comm., anterior commissure.

of these behavioral measures equally. Even though heterosexual copulation was reduced or eliminated, masturbation was apparently undisturbed, since the occurrence of seminal material in the home cage drop pans and the frequency of video-taped masturbation by lesioned males were equal to that of sham-lesioned males. The difference in the effects of MP-AH lesions on masturbation and heterosexual copulation suggests that the MP-AH area is specifically involved with sexual behavior directed towards a partner and is not a neural locus for all forms of sexual behavior.

Recovery of function with regard to copulatory performance in lesioned subjects was observed during the second through fifth months in 4 of the 6 males, varying from 100% in one male to 5% of all tests in another. The level of recovery of ejaculatory ability appeared to stabilize by the end of the sixth month and no more recovery as apparent at 16 months, suggesting that the degree of remaining deficit was permanent. These observations on impairment of copulatory behavior by MP-AH lesions are consistent with those from rats (9, 10, 15), cats (14) and dogs (13). In all species studied some animals have shown partial or complete recovery of copulatory behavior and others

have undergone permanent elimination of copulatory ability.

An analysis of the effects of the MP-AH lesions on heterosexual behavior revealed that components of sexual behavior such as intromission and ejaculation, which occur after contact and mounting, were more reduced than the earlier components. Normally, the number of tests with ejaculation are less than the number of tests with intromission, and those with intromission less than those with mounts or contacts. As shown in figure 77.2, this trend was greatly enhanced by MP-AH lesion. The lesioned males also failed to engage in a second copulatory series when they did ejaculate. These data illustrate that often a copulatory series was begun but not carried through. Whenever ejaculation did occur postoperatively in lesioned subjects with an impairment in sexual behavior, there were significantly more contacts, mounts and intromissions prior to ejaculation and a longer intromission and ejaculatory latency than in sham-lesioned subjects, suggesting that more sexual stimulation was required for lesioned males to attain ejaculation. This effect is in agreement with a recent report (10) that, in male rats, MP-AH lesions interfere with the copulatory mechanism as well as the mechanism for initiating sexual behavior.

Although the copulatory responses of mounting, intromission and ejaculation were altered by MP-AH lesions, yawning during mating, which is also a sexually dimorphic behavior (12), was not affected. Thus it is evident that the lesion did not reduce or impair sexually dimorphic behavior in general.

#### Comparison with Effects of Castration

Serum levels of testosterone in MP-AH-lesioned males were monitored at periodic intervals by radioimmunoassay. The levels of testosterone in MP-AH-lesioned males were found to be comparable to sham-lesioned males (unpublished observations). Thus the changes in sexual activity cannot be explained by interference with the gonadotropin control of testosterone secretion. It is possible that the lesions did destroy androgen sensitive neurons in the basal forebrain, resulting in a type of functional castration. Therefore, a comparison with the effects of castration is of interest.

Castration in the rhesus results in a variable but gradual decline in intromissive and ejaculatory performance. Some animals, though continuing to mount, are apparently incapable of intromission or ejaculation (35). Yawning during mating tests also declines, as well as does the occurrence of ejaculatory plugs in the home cage (33, 35). Direct observation of masturbation frequency has not been made in the studies involving castration, and the disappearance of seminal material from the drop pans could reflect just the cessation of

secretion of seminal material from accessory sexual organs.

In contrast to castration, MP-AH lesions greatly reduced mounting and eliminated or reduced intromission and ejaculation as soon as subjects were tested postoperatively. Importantly, yawning was not affected by MP-AH lesions. One would not expect to see masturbation continue in castrated animals as it did in lesioned subjects, but there are no direct observations to support this. The behavioral effects of MP-AH lesions are therefore not what one would expect from a type of functional castration resulting either from interference with gonadal androgen secretion or from removing some androgen target tissue in the basal forebrain.

#### Social Behavior

In lesioned animals there were no increases in the frequency of threat display or aggression and grooming actually increased. Low frequencies of aggression and the presence of grooming behavior are considered characteristics of stable social interactions (20, 25). The continuing interaction with partners by the lesioned males indicates that sexual deficits were not secondary to some gross social maladjustment. The subjects continued to lever press for access to females, which presumably indicated a predisposition for social interactions with another animal. Analysis of lever pressing tests revealed no preference for the EB-treated females by either sham or lesioned males. The continuation of positive social interactions in the subjects of the present study contrasts with the signs of general social withdrawal in rhesus monkeys following amygdaloid lesions (18).

The present study was not specifically designed to test the effects of MP-AH lesions on female sexual responses, but some interesting observations were recorded. Presenting, which is a part of the male rhesus monkey's behavioral repertoire (11), is normally displayed to other males; presenting is rarely displayed to females. The subjects of this study were not paired with other males, and the occurrence of presenting therefore depended upon the degree to which this behavior was displayed to females, especially when the female partner contacted or attempted to mount the male. In contrast to sham-lesioned subjects, 5 lesioned males (all except the one with complete recovery of copulatory behavior) showed an increase in the number of contacts by the female and the number of presentations when contacted. The presenting by the lesioned males and subsequent mounting by female partners could have been due to an increased tendency of the males to assume a feminine role. However, it is possible that these female partners were using contact and mounting

behavior as a type of sexual solicitation for these relatively sexually inactive males (28).

#### Analysis of Lesions and Recovery of Function

The most effective anterior-posterior locus for the impairment of heterosexual behavior is best described as the junction of the medial preoptic and anterior hypothalamic area at the posterior edge of the anterior commissure (figure 77.5). In the dorsoventral plane, lesions located anywhere between the optic chiasm and the anterior commissure had permanent behavioral effects.

This lesion site is in the same area which has resulted in marked reduction or elimination of copulatory behavior in rats, cats and dogs. In fact, the same interior brain landmarks (posterior edge of the anterior commissure, the region between the anterior commissure and optic chiasm and the medial tissue along the walls of the third ventricle), as revealed by X-ray ventriculography, were found to be the most effective lesion sites for eliminating copulatory behavior in cats (14) and dogs (13). As in the previous work on rats, cats and dogs, large MP-AH lesions were the only ones resulting in elimination of copulatory responses.

The variability in lesion effects could be related to differences in vertical placement of the lesions, rather than minor differences in anterior-posterior or lateral placement, or to the volume of the lesioned area. The three most effective lesions were the three largest, and in the male with no long-term behavioral deficits it was the smallest. While the variability in lesion effects could be explained on an anatomical basis, another possible explanation is that individual differences in preoperative copulatory performance may have determined postoperative performance. The male with the best preoperative copulatory performance, in terms of numbers of ejaculations and the shortest latency to ejaculation, was the male showing the least deficit following MP-AH lesion. The two males with the longest latency to ejaculation and the fewest number of ejaculations preoperatively were the two males with no postoperative ejaculations. Hence, there was a tendency for the degree of preoperative copulatory performance to be related to the effectiveness of the lesion.

In the present study animals were tested 16 months after surgery, but recovery of function only occurred between the second and fifth months. The absence of any further recovery beyond the sixth postoperative month indicates that the MP-AH lesion effect on sexual behavior is permanent once initial recovery occurs. This lack of recovery is also evident in recent work on male rats where the effects of MP-AH lesions are evident within about one month after lesioning with no further recovery apparent for as long as 8 months after surgery (10).

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## Introduction

Since the majority of heterosexual and homosexual individuals do not show consistent differences in peripheral hormone levels (Meyer-Bahlburg, 1977, 1979, in press), it is unlikely that sexual orientation is related to endocrine differences in adulthood. A potential contribution of prenatal hormones to the development of sexual orientation has not been ruled out and therefore has become a focus of current psychoendocrine research. This approach, which is particularly represented by Dörner (1968, 1976), is derived from research on lower mammals for which, regardless of genetic sex, exposure to sex hormones during certain sensitive periods of early brain development leads to structural changes of the brain and specific shifts in subsequent sex-dimorphic behavior and gonadotropin regulation. Two major pathways seem to be involved (McEwen, 1983): an androgenic one that mainly utilizes testosterone and/or DHT, and an estrogenic one that relies primarily on estradiol derived from testosterone by aromatization at the target organ cellular level. Estradiol is widely available in the fetoplacental unit of the rat; it seems to be rendered relatively inert by binding to  $\alpha$ -fetoprotein and has, therefore, effects on sex-dimorphic behavior only when given in pharmacological doses (Plapinger and McEwen, 1978). The non-steroidal synthetic estrogen diethylstilbestrol (DES) is of particular interest since it does not bind to  $\alpha$ -fetoprotein and is able to reach the brain in a biologically active form; there it exerts organizational (masculinizing/defeminizing) effects similar to those of androgens converted to estrogens (Plapinger and McEwen, 1978). Indeed, recent studies—as yet unreplicated—have shown that pre- or perinatal DES alters features of sex-dimorphic childhood social play in female rats (Hines et al., 1982a), increases masculine mounting behavior and decreases feminine lordosis in adult female guinea pigs (Hines et al., 1982b), and leads to the expected paradoxical effect of decreased mounting and intromission behavior in adult male rats (Monroe and Silva, 1982). It also affects the timing of puberty in female rats (Slaughter et al., 1977). Döhler

et al. (1982) have shown structural effects of DES in the preoptic area of the female rat brain.

Data on the effects of prenatal or perinatal hormones on subhuman primates are scant. In prenatally androgenized female rhesus monkeys, the expected effects of prenatal androgens on the development of sex-dimorphic social play behaviors in childhood have been found (Goy and Resko, 1972). The same animals showed increased aggression (and, after testosterone priming, increased proximity toward stimulus females) between 5 and 7 years of age, but their mounting rate did not differ from control females, and only one animal out of seven showed the full masculine copulatory sequence (Eaton et al., 1973). Finally, at the more mature age of 15–17 years, these monkeys gave little evidence of masculinization in their sex-behavioral responsiveness to testosterone (Phoenix and Chambers, 1982). Also, early hormonal influences on gonadotropin regulation in adulthood appeared to be much weaker than in lower mammals (Steiner et al., 1976). A recent study of a new sample of prenatally androgenized female rhesus monkeys (Thornton and Goy, 1983) showed some degree of defeminization in the sexual interaction of these monkeys with males. Due to the small number of monkeys with prenatal hormone treatment investigated in these studies, these findings have to be considered preliminary.

Behavioral studies of prenatal or perinatal DES exposure in nonhuman primates are not available. It has recently been demonstrated (Slikker et al., 1982) that in rhesus monkeys substantial amounts of DES reach the fetal compartment unaltered, in contrast to natural estradiol. Of potentially great significance is another recent study (Fuller et al., 1981) that shows lasting effects of prenatal DES exposure on gonadotropin patterns of infant rhesus monkeys; it is the first study suggesting direct organizational effects of prenatal DES on sex-dimorphic areas of the primate brain.

The generalizability of animal behavior to human analogues, in particular in relation to sexual orientation, is very controversial (Beach, 1979). Nevertheless, that humans are not exempt from prenatal hormone effects on sex-dimorphic behavior has been shown in

previous studies using two paradigms, spontaneous endocrine abnormalities of the fetus and sex hormone treatment during pregnancy (Ehrhardt and Meyer-Bahlburg, 1981; Money and Ehrhardt, 1972). Prenatal androgenization of genetic females shifts many childhood behaviors toward the masculine pole (e.g., Ehrhardt and Baker, 1974; Reinisch, 1981) and may slow down the attainment of psychosexual milestones (Ehrhardt, 1979; Money and Schwartz, 1977; Schwartz and Money, 1983). Hypoandrogenization of males leads to behavior alterations compatible with the animal studies, but the hormonal effects usually cannot be separated from the social effects, since most of the data are based on severe forms of intersexuality (Meyer-Bahlburg, 1977). Nonandrogenizing progestogens administered either alone or in combination with estrogens support the notion of demasculinization of childhood behavior in general (Ehrhardt et al., 1977, 1984; Meyer-Bahlburg et al., 1977) and specifically of aggression (Meyer-Bahlburg and Ehrhardt, 1982; Yalom et al., 1973).

If prenatal hormones play any part in the development of sexual orientation, evidence needs to be presented that individuals who differ only in levels of prenatal hormones differ in adult sexual orientation. Apart from a number of small-scale uncontrolled studies suggestive of such hormonal effects (reviewed in Meyer-Bahlburg, 1977, 1979, *in press*), only one recent study in females employed a clinical group design: Schwartz and Money (1983) have presented data showing increased bisexuality and homosexuality in gonadally intact females with congenital adrenal hyperplasia, a prenatally virilizing condition, when compared to women with vaginal atresia or androgen insensitivity. The only available controlled study that evaluated sexual orientation in males with an unusual prenatal hormone history, namely prenatal DES exposure, did not find a shift in sexual orientation (Kester et al., 1980).

The current study examines the influence of prenatal DES exposure on sexual orientation in females. The working hypothesis to be tested is that prenatal exposure to DES increases the rate of bi- or homosexuality in women as compared to nonexposed controls.

## Methods

### Sample Selection

This investigation was approved by the relevant institutional review board of the first author's institution; the Code of Ethics of the World Medical Association was adhered to. All participating subjects gave written informed consent.

From the DES-screening clinic of one of the authors (N.P.V.), we selected women (DES subjects) aged 17–

30 years whose prenatal DES exposure had been documented by physician, hospital, or pharmacy records. All of them were followed because of the known increased risk of adenocarcinoma of the vagina in female offspring of women who were treated with DES during pregnancy (Herbst et al., 1971) and because of DES-related vaginal or cervical abnormalities such as adenosis (glandular epithelium or its mucinous products in the vagina). Excluded were subjects of races other than Caucasian or socioeconomic status other than middle or upper class (since these were the most frequent demographic background variables of the entire DES clinic population); and subjects with mental retardation; severe congenital abnormalities and debilitating chronic diseases likely to interfere with everyday functioning; and carcinoma. Once target subjects were identified, we spent a great deal of effort in locating subjects who had changed their addresses and enrolling them in the study, even when they were living far away, in order to minimize volunteer effects and related selection biases. Originally, a total of 76 women with prenatal exposure to DES were sent invitations to participate. Of these, 2 could not be located, 2 did not respond to mail or phone contacts, 2 had to be excluded because they did not meet the criteria, 12 refused participation, 4 were undecided, and 54 agreed to participate. The current report concerns the first 30 DES women from this source who completed the research protocol. The data collection is continuing on the remainder of the sample.

Of the first 30 DES women, 15 had sisters who were not prenatally exposed to DES. One was excluded because of young age (10 years). We obtained permission to approach all other sisters for study participation. Where a DES subject had more than one unexposed sister, the one closest in age to the DES subject was selected. Twelve were interviewed. Two refused participation, one because she anticipated psychological stress, the other because of time pressures; both were never married and were younger than their DES sisters.

The DES subjects were to be compared to a second control group of women who were closely matched in age and socioeconomic background and who shared some of the gynecological procedures they had to undergo in adulthood and in the identification as having an increased cancer risk of the sexual or reproductive organs, but who differed in prenatal DES exposure. To obtain such a comparison sample, we selected from the same coauthor's (N.P.V.) private office population women who had been referred for a diagnostic workup because of an abnormal Pap smear (PAP subjects), using identical exclusion criteria. Initially, 104 PAP subjects were selected and contacted either by mail or in person. Of these, 14 could not be located, 17 failed to respond to repeated mailings and phone calls, and

12 had to be excluded on the basis of race or nationality. Of the remaining 61 PAP controls, 41 agreed to participate, 3 were undecided, and 17 refused. There were 32 PAP controls who were interviewed, and of these 2 had to be excluded, one because she failed to meet the socioeconomic criterion and the other because she believed she had cancer.

While definitive documentation of prenatal DES exposure existed for all DES subjects, the prenatal records varied greatly in the details of the information provided. Of 17 subjects on whom the records were sufficiently detailed, two were exposed to DES during the first trimester only, 11 during the first and second trimesters, 3 during the second and third trimesters, and 1 throughout the pregnancy. Of the remaining 13 subjects, 5 were definitely exposed during the first and possibly during the subsequent trimesters, 4 definitely during the second and possibly during the third, 1 definitely during the second and third and possibly during the first trimester. No specific trimester information was available for 3 subjects. Daily dosage was extremely variable and was often systematically increased during pregnancy; the range was 5–250 mg. Total dosage throughout pregnancy (minimum estimate) could be calculated with reasonable certainty for 13 subjects and ranged from 210 to 10,475 mg. The fact that 27 (90%) of our DES subjects had vaginal adenosis—a much higher percentage than is usually found in DES-exposed women (O'Brien et al., 1979)—makes it likely that our sample was exposed to DES earlier, longer, and to higher dosages than DES women in general. Of the DES subjects, 25 had been exposed to DES as the only hormone; additional hormones the subjects were exposed to were progesterone in 3 cases; anhydrohydroxy-progesterone (Prenone) in 1 case; and progesterone plus estradiol in 1 case. With regard to the 12 sisters, nonexposure to DES was documented by medical records for 10; the records of the remaining two could not be retrieved, but their mothers were sure that they had not been treated with DES or any other hormone during those pregnancies.

The 30 PAP subjects had undergone colposcopy and, if indicated, punch biopsy, rendering diagnoses of mild, moderate, or severe dysplasia in 22 cases. Many of these had been treated with cryosurgery or cone biopsy, depending on the severity of dysplasia, although some required no treatment at all. In 8 cases, colposcopic examination or biopsy did not reveal any dysplasia, and the abnormal Pap smear may have indicated an inflammation or an infection. Although documentation of prenatal hormone exposure could not be obtained for this sample, exposure to DES is rather unlikely since none of the subjects or their mothers knew about such exposure, nor did any of the PAP subjects show vaginal adenosis.

### Assessment Methods and Procedures

All subjects selected were contacted by their gynecologist (N.P.V.) with a letter explaining the purpose of the study as the psychological assessment of DES patients/PAP patients with the goal to ascertain general psychological development and specific emotional reactions to their medical history. A questionnaire (with return envelope addressed to the gynecologist) was included on which the patients were to indicate whether or not they were interested in study participation. Return of the questionnaire was rewarded with a \$3 check. Subsequently, all interested patients were contacted for scheduling. Once a patient agreed to participate in the study, we asked her permission to contact her mother for participation in the project and, where available, the unexposed sister closest in age. Mothers and sisters were then contacted in the same way. Most subjects were evaluated in our research unit at Columbia; some preferred to be seen in the office suite of their gynecologist (N.P.V.), and some had to be tested and interviewed during a home visit. Subjects were reimbursed for their travel expenses and received an additional bonus of \$35 for participation.

The 8-hour evaluation protocol included a variety of psychiatric and psychological assessment methods. Psychosexual development and functioning, the main focus of this report, were evaluated by the Sexual Behavior Assessment Schedule-Adult<sup>1</sup> (SEBAS-A; see Meyer-Bahlburg and Ehrhardt, 1983), which covers psychosexual milestones (of romanticism and sexual behavior), sexual orientation, sexual activity level, and sexual dysfunctions. Sexual orientation in terms of imagery, attractions, and overt sociosexual behavior was assessed both for the 12 months preceding the examination and for the interval from puberty to the present ("lifelong"). Aspect-specific subscales as well as global scales were used and included the following variables: masturbation fantasies, masturbation erotica, romantic/sexual daydreams, romantic/sexual nightdreams, sexual attractions, imagery (a global rating encompassing all previously listed variables with the exception of masturbation erotica), sexual relations (with partners), and sexual responsiveness (a global rating incorporating all variables).

For each subscale and each global scale, the Kinsey Rating Scale (Kinsey et al., 1948, 1953) was used with the following formulation: 0 = entirely heterosexual; 1 = largely heterosexual but incidentally homosexual (e.g., night dreams, masturbation fantasies); 2 = largely heterosexual but also distinctly homosexual; 3 = equally heterosexual and homosexual; 4 = largely homosexual but also distinctly heterosexual; 5 = largely homosexual but incidentally heterosexual; and 6 = entirely homosexual. Kinsey 2, or "a distinct" homosexual history was rated when the subject had an

**Table 78.1**  
Sample characteristics of DES and PAP subjects

	DES ( <i>N</i> = 30)			PAP ( <i>N</i> = 30)			<i>t</i> Test ( <i>p</i> , 2-tailed)
Characteristic	<i>N</i>	Mean	S.D.	<i>N</i>	Mean	S.D.	
Age at interview (years)	30	25.13	3.74	30	26.00	3.25	n.s.
Subjects' Hollingshead 4F Index	22 <sup>a</sup>	50.93	9.27	27 <sup>a</sup>	48.56	10.84	n.s.
Parents' Hollingshead 4F Index	29 <sup>b</sup>	49.78	9.89	30	45.13	10.28	n.s.
WAIS-R Full IQ	18 <sup>c</sup>	105.94	9.79	30	98.37	10.21	≤ .015
	<i>N</i>	%		<i>N</i>	%		$\chi^2$ ( <i>p</i> , 2-tailed)
<i>Current marital status</i>							
Married <sup>d</sup>	11	36.7		19	63.3	}	≤ .100 <sup>e</sup>
Divorced	4	13.3		3	10.0		
Never married	15	50.0		8	26.7		
<i>Current religion</i>							
Catholic	5	16.7		13	43.3	}	< .100 <sup>f</sup>
Protestant	2	6.7		3	10.0		
Jewis	20	66.7		13	43.3		
Other	3	10.0		1	3.3		

<sup>a</sup> The remaining subjects were still students and unmarried at the time of the interview.

<sup>b</sup> This index could not be computed for one DES-exposed subject, due to missing information.

<sup>c</sup> One DES woman was not tested with either the WAIS or WAIS-R because she was familiar with both tests. This subject received the Primary Mental Abilities Test, instead; her full IQ from this test was 126. Eleven DES women were tested with the WAIS (Wechsler, 1955) before the WAIS-R (Wechsler, 1981) became available: Mean Full IQ = 113.00, S.D. = 6.50. According to Wechsler (1981), a WAIS Full IQ of 113 is approximately equal to a WAIS-R Full IQ of 105; thus, the two DES subgroups are comparable in level of intelligence.

<sup>d</sup> One of the married PAP women was separated.

<sup>e</sup>  $\chi^2$  was calculated for married plus divorced vs. never married DES and PAP women.

<sup>f</sup>  $\chi^2$  was calculated after collapsing "Protestant" and "Other."

activity such as homosexual dreams over a period of at least 1 year recurring with some regularity rather than sporadically or incidentally. Whenever a subscale was rated 2, the corresponding global score could not be rated less than 2. In a few cases, global ratings were rated down to 0 when the highest subscale rating was a marginal Kinsey 1. Additional items concerned homosexual milestones, number of partners, frequency of heterosexual versus homosexual orgasmic experiences, etc.

All SEBAS-A interviews were conducted by female interviewers. Due to staff shortages, on the one hand, and frequent self-disclosure of the patients, on the other, only a fraction of the interviews could be conducted blindly. All interviews were audiotaped. For all subjects who were rated other than 0 on any of the Kinsey scales, all Kinsey-format items were independently and blindly co-rated from tape by three co-raters, and disagreements were resolved by discussion. Data analysis was based mainly on statistical case-control comparisons.

## Results

### DES versus PAP Subjects

The sample characteristics are shown in table 78.1. At the time of interview, both groups were approximately the same age. Social status, as reflected in the

Hollingshead Four-Factor Index (Hollingshead, 1975), was also similar for both the subjects and their parents. The Index is based on education and occupation levels and takes marital status into consideration. The majority of both groups are of middle-class or higher socioeconomic background. With respect to the WAIS-R Full IQs, the DES-exposed group was about one-half S.D. above the PAP subjects, a significant difference. (The project was started before the WAIS-R was published. Therefore, the first subjects were tested with the older WAIS form.) Fewer DES women were married than PAP women, a nearly significant difference. There were relatively more Jewish women in the DES-exposed group, and Catholic women in the PAP sample.

The data on sexual orientation are listed in table 78.2 and Appendix A. In comparison with the PAP women, the DES-exposed women had higher Kinsey scores on all items, with most of the differences of at least borderline ( $p < 0.10$ ) statistical significance. Out of the ten variables, only one, masturbation erotica, failed to reach borderline significance, probably due to the relatively small number of women who were using erotic depictions or narratives for masturbatory arousal. The percentages of DES women with bisexual or homosexual responsiveness (Kinsey scale 2 through 6) was 21% (6 out of 29 women with data on this issue) for the preceding 12 months and 24% (7 women) for

**Table 78.2**DES vs. PAP group comparisons: Kinsey scales (Mann-Whitney *U* test)

	<i>N</i>		Direction of Difference <sup>a</sup>	<i>p</i> (1-Tailed)
Variable	DES	PAP		
<i>Current (past 12 months)</i>				
Masturbation fantasies	18	18	↑	.020
Masturbation erotica	8	6	↑	n.s. <sup>b</sup>
Romantic/sexual daydreams	22	29	↑	.063
Romantic/sexual nightdreams	13	21	↑	.068
Sexual attractions	29	28	↑	.003
Global rating: imagery	28	30	↑	.094
Sexual relations	28	30	↑	.017
Global rating: sexual responsiveness	29	30	↑	.044
<i>Lifelong (since establishment of a sex life)</i>				
Sexual relations	29	30	↑	.018
Global rating: sexual responsiveness	29	30	↑	.004

<sup>a</sup>↑, Kinsey scores of DES subjects are higher than those of controls.<sup>b</sup>n.s.,  $p \geq .100$ .

lifelong, as compared to 3% (one subject) for the preceding 12 months and none for lifelong among the PAP women (Fisher's Exact Test, one-tailed,  $p < 0.05$  and  $p < 0.01$ , respectively).

Five of the 29 DES women, or 17%, had a Kinsey score of at least 3 (i.e., at least equally homo- and heterosexual) for overall sexual responsiveness during the preceding 12 months compared to none in the PAP sample, also a statistically significant result (Fisher's Exact Test,  $p < 0.05$ , onetailed).

Other sections of the interview revealed that six of the seven DES women with a Kinsey score of 2 and higher for lifelong sexual responsiveness had had romantic friendships with other women but that only five had the experience of overt homosexual activities involving genital contact. During the 12 months preceding the examination, two were living with a homosexual partner. For one subject, with a Kinsey 3 rating, the bisexuality was limited to romantic and erotic imagery including attractions and crushes but had not been expressed in mutual romantic or sexual relationships with other women.

#### DES Subjects and Sister Controls

Those DES-exposed subjects for whom DES-unexposed sisters were available were separately analyzed in comparison to their control sisters. The sample characteristics of the resulting 12 sibling pairs are listed in table 78.3. DES-exposed sisters and controls were not significantly different in terms of age, their own or their parents' socioeconomic status, or intelligence. However, significantly fewer of the DES subjects were ever married than subjects of the sister group.

Table 78.4 and Appendix B show the results on sexual orientation. As in the case of the DES-PAP comparisons, the DES women had higher scores on all

10 Kinsey variables than their nonexposed sisters, although only a few of these (sexual attractions, global rating of sexual responsiveness in the previous 12 months, and global rating of sexual responsiveness lifelong) reached statistical significance. Five of the 12 DES women (42%) had a sexual responsiveness rating of 2 or higher both for the 12 months prior to the examination and lifelong, as compared to one (8%) of the 12 nonexposed sisters ( $p \leq 0.062$ , 1-tailed, Binomial Test); and three of the DES-exposed women (25%) had scores of 3 and higher for the previous 12 months and two (17%) for lifelong, as compared to none of their nonexposed sisters ( $p \leq 0.125$  and  $p \geq 0.200$ , respectively, 1-tailed, Binomial Test).

#### Discussion

The results of this study show a significantly increased rate of bi- or homosexuality in DES-exposed women compared to two independent control groups. There is a high consistency of this finding across a number of different Kinsey-scale rating categories, and the ratings were confirmed by multiple raters.

Before concluding that the findings on sexual orientation are related to prenatal DES, one has to consider a number of other possibilities. One issue to consider is sampling bias. It is unlikely that any systematic bias favoring bi- or homosexual individuals was introduced in the subject identification procedures, since they were based exclusively on the availability of documentation of DES exposure and on the demographic characteristics listed above. If there was a volunteer effect of the kind that people who were earlier or more easily enrolled in the study tended to be more frequently bi- or homosexual, these biases should have affected the two control samples in the same direction. On the other

**Table 78.3**

Sample characteristics of paired subjects and sister controls

Characteristic	DES ( <i>N</i> = 12)			Sisters ( <i>N</i> = 12)			Paired <i>t</i> Test ( <i>p</i> , 2-Tailed)
	<i>N</i>	Mean	S.D.	<i>N</i>	Mean	S.D.	
Age at interview (years)	12	24.50	3.70	12	23.58	4.08	
Subjects' Hollingshead 4F Index	8 <sup>a</sup>	50.75	8.94	7 <sup>a</sup>	45.29	13.14	
Parents' Hollingshead 4F Index	12	53.88	9.24	12	53.88	9.24	
WAIS-R Full IQ	8 <sup>b</sup>	110.88	8.87	8 <sup>b</sup>	104.62	11.45	
	<i>N</i>	%		<i>N</i>	%		$\chi^2$ (McNemar) ( <i>p</i> , 2-Tailed)
<i>Current marital status</i>							} $\leq .001^d$
Married <sup>c</sup>	4	33.3		8	66.7		
Divorced	0	0.0		0	0.0		
Never married	8	66.7		4	33.3		
<i>Current religion</i>							
Catholic	2	16.7		3	25.0		
Protestant	1	8.3		2	16.7		
Jewish	7	58.3		7	58.3		
Other	2	16.7		0	0.0		

<sup>a</sup> The remaining subjects were still students and unmarried at the time of the interview.<sup>b</sup> Three DES women were tested with the WAIS resulting in a mean Full IQ of 114.67, S.D. = 4.51, corresponding to a WAIS-R Full IQ of about 107. (An additional DES woman was tested with the PMA). The mean WAIS-R Full IQ of the three sister controls whose DES-exposed sisters were tested with the WAIS was 104.35. This difference is not significant, nor is the difference between the eight pairs of WAIS-R tested women.<sup>c</sup> One of the married sister controls was separated.<sup>d</sup>  $\chi^2$  was calculated for married vs. never-married DES women and their sisters.**Table 78.4**

DES vs. sister group comparisons: Kinsey scales (sign test)

Variable	<i>N</i> (pairs)	Direction of Difference <sup>a</sup>	<i>p</i> (1-Tailed) <sup>b</sup>
<i>Current (past 12 months)</i>			
Masturbation fantasies	4	↑	n.s.
Masturbation erotica	2	↑	n.s.
Romantic/sexual daydreams	8	↑	n.s.
Romantic/sexual nightdreams	3	↑	n.s.
Sexual attractions	12	↑	.062
Global rating: imagery	11	↑	n.s.
Sexual relations	11	↑	n.s.
Global rating: sexual responsiveness	12	↑	.032
<i>Lifelong (since establishment of a sex life)</i>			
Sexual relations	12	↑	n.s.
Global rating: sexual responsiveness	12	↑	.016

<sup>a</sup> ↑, Kinsey scores of DES subjects are higher than those of controls.<sup>b</sup> n.s., *p* ≥ .100.

hand, are bisexual or homosexual individuals underrepresented in the control samples? For instance, in the case of the PAP group, one could speculate that this is a hyperheterosexual sample, since early onset of sexual experience and a history of multiple partners have been linked to an increased risk of developing abnormal Pap smears (Rotkin, 1973). The association between early onset of sexual intercourse and the development of cervical cancer or cervical dysplasia as diagnosed by an abnormal Pap smear is, however, mainly true for women of lower SES (Rent et al., 1972), which does not apply to our sample. In order to get a population estimate of behavior frequencies of the various Kinsey scores, we combined the corresponding Active Incidence data for ages 25 and 30, separately for married and for single plus previously married females (Kinsey et al., 1953, p. 499, table 142) and applied these figures to all samples, calculating expected values separately for married and for divorced plus never married women, and then combining the figures for the total sample. On this basis, the expected number of persons with a Kinsey score 2 to 6 in the PAP sample is 1.3, and in the sister sample 0.5; the expected value for Kinsey score 3 to 6 for the PAP sample is 0.8, and for the sister sample 0.3. The actual numbers of such people found in the control groups are very close to these expected values. Thus, these low rates by themselves do not indicate an underrepresentation of the bi- and homosexual spectrum. If one applies the same calculations to the DES sample, the expected numbers for Kinsey 2 to 6 individuals is 2.1, and for Kinsey 3 to 6 individuals, 1.3. The numbers of bi- or homosexual DES-exposed women found in our sample are considerably higher, corroborating the conclusion that bi- and homosexuality are truly increased in the DES sample. Moreover, the expected values calculated for the DES sample exceed those expected for the population at large (which has a lower percentage of singles in this age group than in our DES sample) so that our excess findings are very strong. Of course, the comparison to possible outdated population norms is problematic and has to be considered a crude approximation.

Sampling bias could also play a role if subject and control groups differ in some important demographic variable. However, the DES and PAP groups are very similar in socioeconomic levels and age at examination. The demographic group differences observed, such as in student status, Wechsler IQ, number of siblings, or religion are not large and are unlikely to account for the difference in sexual orientation, since there is little or no evidence in the literature of an association of these variables with sexual orientation in women. With regard to current religion, the seven bi- or homosexual DES women break down as follows: 4

are Jewish, 1 Protestant, 1 Catholic, and 1 other (raised Protestant). Both the PAP woman and the unexposed sister with Kinsey 2 scores are Protestant. Thus, in comparison to the distribution of religion in the total samples, there does not seem to be a particular religious bias associated with Kinsey 2–6 scores. It is also important to note that the demographic differences do not hold up for the DES women in comparison to their unexposed sisters.

If sampling bias is excluded as a confounding variable, a nonhormonal psychological explanation of our findings also has to be considered. The most plausible factor for the increased bisexuality might be the awareness of being a DES daughter. One could hypothesize that DES women might be at risk for being discouraged from heterosexuality because they are more likely to expect sexual and reproductive problems stemming from their history of DES exposure. We are not aware, however, of any systematic data supporting such an association for other medical syndromes. For instance, conditions with an extremely low rate of fertility, such as Turner syndrome, do not seem to be associated with increased bisexual or homosexual orientation. In our syndrome-specific interview schedules, we asked about the impact of their respective medical conditions on various areas of life. Only two of the 30 DES women mentioned worries about their sex life, in one case continuing to the time of evaluation; both were heterosexual. Thirteen mentioned current concerns about their ability to bear children; two of these belonged to the group of seven bi- or homosexual women. None of the seven stated spontaneously that awareness of the DES condition was responsible for their bisexual development. A definitive answer to this issue cannot be given by our data, however, because none of the interview schedules addressed this particular issue in detail.

Another psychological theory (Storms, 1981) suggests that bi- or homosexual orientation is more likely to develop when puberty starts relatively early. This reasoning does not apply to our samples since there was no significant difference between DES and PAP women nor between DES women and their unexposed siblings, in the recalled age at menarche; on average, the DES women were slightly later than the controls (Meyer-Bahlburg et al., 1984).

Concerning possible psychoendocrine interpretations of our findings, both adult and prenatal hormone conditions must be considered. Two recent endocrine studies of clinical samples of DES-exposed women (Peress et al., 1982; Wu et al., 1980) have demonstrated markedly increased rates of hirsutism and irregular menstrual cycles, both likely to be related to androgens. In fact, the same reports showed elevated testosterone levels in the DES women, especially in those

with hirsutism. The testosterone levels were similar to those of hirsute women without a history of DES exposure. In this context, it is interesting to note that elevated androgen levels—although not characteristic of the majority—have been found in about one-third of the homosexual women on whom endocrine data have been published (Meyer-Bahlburg, 1979). Androgens have repeatedly been related to various aspects of female sexuality, and Sanders and Bancroft (1982) have suggested that a negative correlation of testosterone level and satisfaction with heterosexual relationships may lead some women with relatively high testosterone levels into adopting homosexual life-styles. Since, on the other hand, data on positive correlations of androgens and female sexual interest predominate, one can also hypothesize that increased sexual motivation may lead to an enhanced sexual responsiveness to a wider range of stimuli, including those of the same sex (via generalization), both in imagery and overt behavior. This reasoning, however, does not apply to our samples, since the DES women appear to be lower in sexual motivation than the PAP subjects (Meyer-Bahlburg et al., in press). Hormone assays were not part of the present project; thus, we cannot ascertain the potential contributions of sex hormone level differences to our findings.

If data from animal research apply to the human condition, an endocrine contribution during very early (prenatal or perinatal) phases on sex dimorphism of brain and behavior is more likely. Available data on adult women with prenatal androgen excess either only prenatally (Schwartz and Money, 1983) or pre- and postnatally (Ehrhardt et al., 1968) demonstrate increased rates of bi- and homosexuality similar to the ones found in our DES sample. With regard to the question of which hormonal pathway might be involved in these presumptive brain effects (McEwen, 1983), our data suggest a participation of the estrogen pathway, which is not at variance with the findings on CAH women, since androgens may easily be aromatized to estrogens at the target organ level. The documentation of dosage available to us on these women does not allow us to attempt any statement on dose-response relationships.

A hormonal interpretation is also suggested by the fact that the seven DES women with Kinsey scores 2–6 were exposed to DES only, whereas the women who had DES plus progesterone exposure were all Kinsey 0–1 heterosexuals. A number of animal studies (e.g., Kincl and Maqueo, 1965; Dorfman, 1967; McEwen et al., 1979) have shown that progesterone may protect against masculinizing/defeminizing hormonal effects—of both androgens and estrogens—during early development.

In CAH women, prenatal androgen excess is associated with marked tomboyish childhood play behavior (Ehrhardt and Meyer-Bahlburg, 1981), which may have some significance in their development of higher rates of bi- and homosexuality later in life. Preliminary analysis of the gender-role behavior data in the current study also shows differences between DES women and their controls, although not in regard to tomboyism. The DES women were less nurturant/maternal throughout their development. An interpretation of these data in relation to the development of sexual orientation is difficult.

From the viewpoint of rigorously controlled animal research, studies of human subjects with prenatal exposure to exogenous sex hormones are methodologically quite problematic. In our study, for instance, the DES subjects went through at least four preselection procedures. (1) Pregnancy treatment with DES was not administered in a random fashion but usually only to mothers with a history of miscarriage or acute pregnancy problems. (2) Our DES daughters were selected from a DES screening clinic. It is well-known that DES women who come to DES screening centers, either on their own or on referral by their physicians, typically have more numerous or more severe medical symptoms of DES exposure than DES women selected at random from pregnancy records (O'Brien et al., 1979). (3) Selected for our study were DES daughters from a screening clinic population on whom prenatal documentation was available. (4) Some of those targeted could not be traced or refused to participate in the study. Steps 1 and 2 affect prevalence rates of medical symptoms. Whereas we are not aware of any evidence that suggests an influence of these selection steps on the prevalence rates of various degrees of sexual orientation, behavioral effects of some kind seem plausible. Steps 3 and 4 were shared between DES subjects and PAP controls, but steps 1 and 2 leave room for unforeseen confounding variables. Clearly, extreme caution in data interpretation is indicated, and replication in other samples with different control groups is required. Therefore, the findings presented here can only be considered suggestive and are not to be taken as proof of a hormonal contribution to the development of sexual orientation in humans. Even if hormones in general, and DES in particular, should have some influence on the development of sexual orientation, it is important to note that 75% of the DES-exposed women in our sample were exclusively or nearly exclusively heterosexual in spite of the DES exposure. Only one woman out of 30 was nearly exclusively homosexual from the establishment of her sexual life. This means that, at best, DES exposure may have contributed somewhat to the development of sexual orientation but does not

have a strong and certainly not a determining influence. Moreover, findings on sexual orientation in individuals with hormonal abnormalities or particular hormonal treatment regimens during fetal life may not have any bearing on the etiology in bisexual or homosexual individuals without such medical histories (Meyer-Bahlburg, in press). Therefore, any conclusions from the data on the specific samples of our study to the development of sexual orientation in general seem unwarranted at this time.

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### Note

1. Copies are available from the authors.

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## Appendix A

DES subjects ( $N = 30$ ) vs. PAP subjects ( $N = 30$ ): number of subjects with individual Kinsey scores (K0–K6)

Variable	DES								PAP							
	K0	K1	K2	K3	K4	K5	K6	NA <sup>a</sup>	K0	K1	K2	K3	K4	K5	K6	NA <sup>a</sup>
<i>Current (past 12 months)</i>																
Masturbation fantasies	10	3	1		1		3	12	15	3						12
Masturbation erotica	4	1	2			1		22	3	3						24
Romantic/sexual daydreams	14	3	1	1	1	1	1	8	23	5	1					1
Romantic/sexual nightdreams	8	2	1		1		1	17	17	4						9
Sexual attractions	20	3		2	1	1	2	1	27		1					2
Global rating: imagery	17	5	2			2	2	2	22	6	2					
Sexual relations	24	1	1				2	2	30							
Global rating: sexual responsiveness	17	6	1	1	1	1	2	1	24	5	1					
<i>Lifelong (since establishment of a sex life)</i>																
Sexual relations	23	1	3		1		1	1	29	1						
Global rating: sexual responsiveness	13	9	3	2	1	1		1	22	8						

<sup>a</sup>NA = Number of subjects for whom the particular variable is not applicable.

**Appendix B**DES subjects ( $N = 12$ ) vs. sisters ( $N = 12$ ): number of subjects<sup>a</sup> with individual Kinsey scores (K0–K6)

Variable	DES							Sisters						
	K0	K1	K2	K3	K4	K5	K6	K0	K1	K2	K3	K4	K5	K6
<i>Current (past 12 months)</i>														
Masturbation fantasies	2		1		1			3				1		
Masturbation erotica	1						1	1			1			
Romantic/sexual daydreams	6			1		1		7	1					
Romantic/sexual nightdreams	1	1	1					3						
Sexual attractions	8			2	1	1		10	1	1				
Global rating: imagery	8		2			1		9	1		1			
Sexual relations	9	1	1					11						
Global rating: sexual responsiveness	7		2	1	1	1		9	2	1				
<i>Lifelong (since establishment of a sex life)</i>														
Sexual relations	9		3					12						
Global rating: sexual responsiveness	6	1	3	2				9	2	1				

<sup>a</sup>For each variable, the frequency is based on the number of matched pairs where each pair member has a Kinsey rating; matched pairs where one or both members have an NA ("Not Applicable") rating are excluded.



## Introduction

The suprachiasmatic nucleus (SCN) of the hypothalamus is a cell group located in the basal part of the mammalian brain (figure 79.1). It is considered to be the principal component of the biological clock generating and coordinating hormonal, physiological and behavioral circadian rhythms (24, 25, 29). In addition it is thought to be involved in reproduction (30, 33). Because of the differences in circadian rhythms found in relation to sex (11, 42) and the attenuation of circadian rhythmicity with aging as well as in Alzheimer's disease (5, 28, 41) the human SCN has been studied with particular reference to these conditions (21, 32, 33). We found, for example, a marked cell loss in the human SCN in late onset Alzheimer's disease (32, 33) dropping to values which were only about 30% of the cell number found in normal adults.

In order to investigate whether cell loss in the SCN also occurs in other types of dementias, we subsequently examined the SCN in postmortem brains of subjects with early onset Alzheimer's disease as well as of patients who died with an AIDS–dementia complex (27). Our studies revealed that the SCN indeed was as strongly affected in early onset as in late onset Alzheimer's disease (19). However, we found that the SCN was not smaller in subjects with AIDS–dementia complex than in the reference group, but rather appeared to be considerably enlarged. Subsequent research, as reported in the present paper, suggested that the enlarged SCN is related neither to the AIDS–dementia complex nor to AIDS per se but rather to homosexuality.

## Materials and Methods

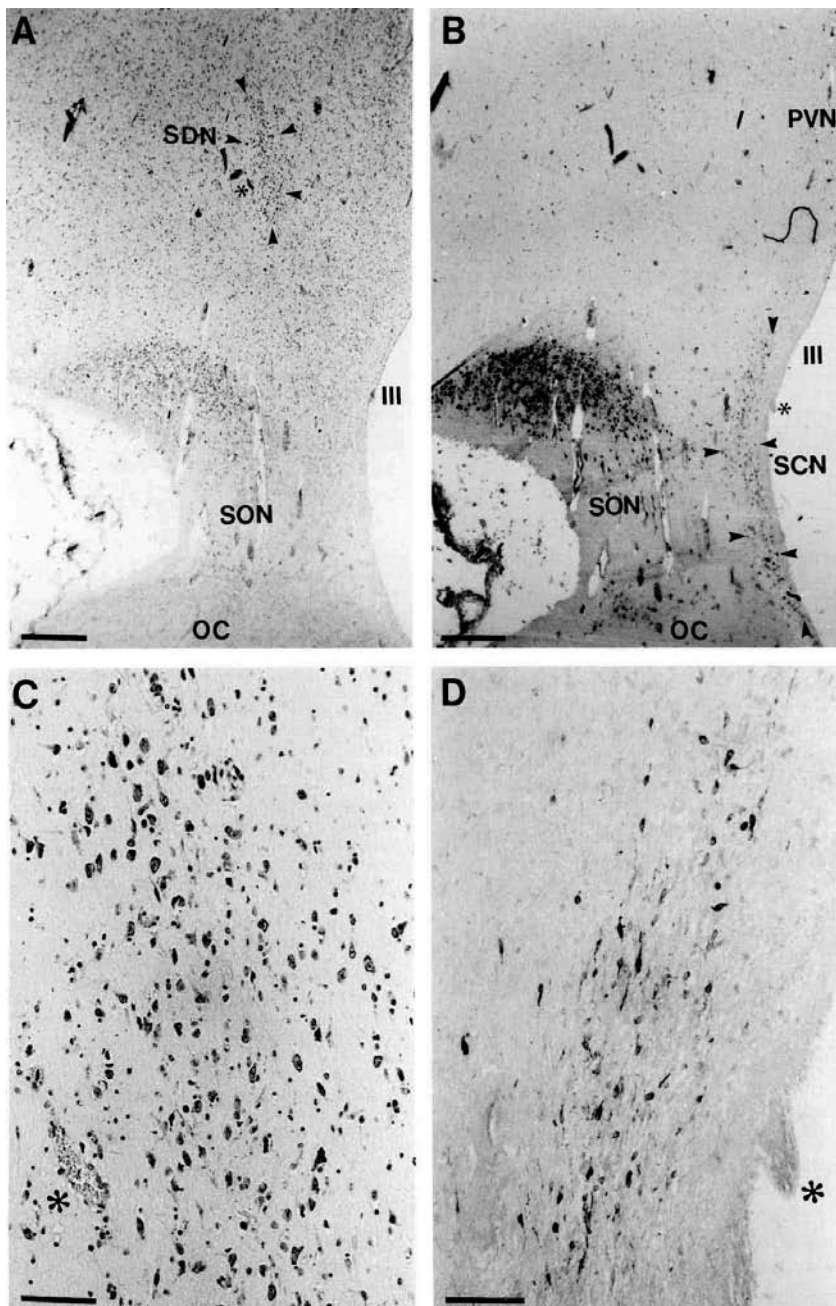
### Subjects

For the present study the brains of 34 subjects were investigated (table 79.1). The required, separate permission for brain autopsy was obtained either from the patients themselves or, in case of dementia, from partners or relatives. The reference group consisted of brains of 18 male subjects from 22 to 74 years of age

( $39.9 \pm 3.6$  years; mean  $\pm$  S.E.M.). General pathology and neuropathology were performed either at the Free University of Amsterdam (Dr. W. Kamphorst) or at the Academic Medical Center of the University of Amsterdam (Dr. D. Troost). Sexual preference of the subjects of the reference group was generally not known. The homosexual male group consisted of 10 non-demented AIDS subjects (aged 25–43;  $36.7 \pm 2.1$  years). AIDS patients were diagnosed according to the Centers for Disease Control (6). As a control group the same parameters were measured in 6 non-demented heterosexuals (4 males, 2 females; aged 21–73 years;  $36.7 \pm 7.5$  years) who also died from AIDS. Two contracted AIDS by blood transfusion, two by sexual contact and two were drug addicts. Excluding the two female subjects from the group of heterosexuals with AIDS, in order to confine the analysis exclusively to male subjects, did not in any way affect the outcomes. Sexual preference of the subjects of the homosexual and heterosexual AIDS groups was registered in the clinical records. Only after the measurements were performed, was it established from the records whether or not the AIDS patients belonged either to the non-demented or demented homosexual groups or to the heterosexual group.

### Histology

Brains were generally weighed after removal followed by fixation in formaldehyde at room temperature. No significant differences in postmortem delay were found among the 3 groups considered ( $P > 0.2$ ). In contrast, the fixation times in the reference group were on average longer than in the homosexual male group ( $40 \pm 3$  days and  $29 \pm 4$  days, respectively,  $P < 0.05$ ). Since the length of the fixation period and the SCN volume and cell number were not found to be correlated ( $P > 0.3$ ) the difference in fixation time between these groups will not affect the outcome. After fixation, the hypothalamic area was dissected, dehydrated and embedded in paraffin. Serial 6  $\mu$ m frontal sections were cut on a Leitz microtome, mounted on chrome-alum-coated slides, hydrated, brought to phosphate-buffered saline (PBS) and each 50th section was stained



**Figure 79.1**

Thionin staining (*A, C*) and vasopressin staining (*B, D*) of the 6  $\mu$ m hypothalamus adjacent sections of patient no. 28. In the overview the SDN (*A*) and SCN (*B*) are indicated by arrowheads. The asterisk marks the site of a blood vessel and ventricular wall that corresponds with the asterisk in the higher magnification (*C* and *D*, respectively). The bars indicate 1 mm (*A* and *B*) or 0.1 mm (*C* and *D*). OC, optic chiasm; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; SDN, sexually dimorphic nucleus of the preoptic area; SON, supraoptic nucleus; III, third ventricle.

**Table 79.1***Brain material*

All patients were male subjects, except for two female patients in the group of heterosexuals who died from AIDS (patient nos. 33 and 34)

Patient Number	Age (Years)	Brain Weight (g)*	Suprachiasmatic Nucleus			Post-mortem Delay (h)	Fixation (Days)	Clinical Diagnosis
			Volume (mm <sup>3</sup> )	Total Cell Number (×10 <sup>3</sup> )	AVP Cell Number (×10 <sup>3</sup> )			
Reference group								
1	22	1570	0.380	46.82	12.93	15	35	Lymphatic leukemia
2	23	1310	0.379	58.98	9.92	13	11	Encephalitis in brainstem
3	27	1330	0.129	17.75	3.45	40	28	Coma
4	27	1560	0.196	22.87	3.97	24	40	Drug addiction; sepsis ( <i>Staphylococcus aureus</i> )
5	28	1510	0.303	40.69	5.45	23	32	Medial cerebral artery aneurysm; vena cave superior syndrome; lung emboli
6	28	1450	0.233	43.27	9.89	24	46	Guillain-Barré syndrome
7	29	1400	0.279	60.51	5.24	13	41	Congenital heart disease; cardiac failure
8	31	1330	0.339	74.99	9.25	29	30	Multiple trauma; small subarachnoidal hemorrhage
9	37	1370	0.133	18.76	1.87	39	35	Bronchopneumonia
10	37	1510	0.169	24.79	2.95	48	46	Alcohol intoxication, combined with benzodiazepines
11	41	1440	0.464	84.55	13.73	120	44	Cerebral contusion, lung emboli
12	42	1510	0.214	54.31	9.60	22	42	Metastatic bronchogenic carcinoma; pneumothorax
13	43	1260	0.195	58.93	10.75	23	53	Non-Hodgkin lymphoma; sepsis
14	47	1620	0.138	24.02	2.24	24	39	Amyotrophic lateral sclerosis/spinal muscular atrophy
15	59	1350	0.260	33.77	6.35	4	53	Pulmonary emphysema; pneumothorax
16	61	1400	0.285	56.97	8.10	22	51	Myocardial infarction; cardiac failure
17	63	1420	0.305	53.38	8.93	32	35	Myocardial infarction; cardiac failure
18	74	1410	0.246	37.00	7.34	13	48	Cardiac failure; bronchopneumonia
Homosexuals (AIDS)								
19	25	1530	0.534	106.75	18.40	47	28	AIDS, pneumonia
20	30	1480	0.669	101.29	21.80	4	31	AIDS, cytomegalic infections
21	30	1640	0.413	59.11	15.57	24	26	AIDS, <i>Pneumocystis carinii</i> pneumonia
22	32	1440	0.473	99.94	12.26	49	11	AIDS, <i>Pneumocystis carinii</i> pneumonia
23	39	>1320	0.432	117.59	10.83	24	28	AIDS, progressive multifocal leukoencephalopathy
24	41	>1240	0.634	147.73	20.61	12	34	AIDS, bronchopneumonia, cytomegalic infections and toxoplasmosis
25	42	1340	0.109	24.66	2.70	4	35	AIDS, disseminated Kaposi sarcoma and generalized mycobacterium avium infection
26	42	1340	0.481	90.91	12.15	19	30	AIDS, cytomegalic meningoencephalitis
27	43	>1260	0.279	56.47	16.94	2	96	AIDS, disseminated Kaposi sarcoma and pneumonia
28	43	>1340	0.375	64.20	10.29	24	17	AIDS, <i>Pneumocystis carinii</i> pneumonia, Kaposi sarcomas, cytomegalic infections
Heterosexuals (AIDS)								
29	21	1500	0.154	36.96	3.35	17	26	AIDS, mycobacterial infections pneumonia, cerebrovascular accident
30	30	1430	0.092	23.82	2.56	8	35	AIDS, <i>Pneumocystis carinii</i> pneumonia, lung tuberculosis, toxoplasmosis, heroin addiction
31	30	1340	0.244	40.77	6.97	8	26	AIDS, disseminated non-Hodgkin lymphoma infections, drug use
32	32	1340	0.128	43.56	1.48	11	131	AIDS, cytomegalic infections
33	34	1400	0.181	40.38	5.14	12	24	AIDS, disseminated histoplasmosis
34	73	>1090	0.258	55.88	4.38	48	38	AIDS, pneumonia, epilepsy

\*Inequality signs indicate that parts of the brain were already removed directly after autopsy before weighing the brain.

with thionin for orientation. Volume and cell numbers were determined in two hypothalamic nuclei, the suprachiasmatic nucleus (SCN) and the sexually dimorphic nucleus (SDN) (22, 32, 34). The SDN (figure 79.1), which is identical with the intermediate nucleus (3) and twice as large in males as in females, was included as a reference nucleus in order to test the hypothesis of 'female differentiation of the hypothalamus' as a biological explanation for male homosexuality (13, 15). The SDN was visualized in thionin-stained sections (31) (figure 79.1) whereas for the SCN vasopressin—one of the main neurotransmitters or neuro-modulators in this structure—was used as a marker (32) (figure 79.1).

### Immunocytochemistry

For immunocytochemistry the hydrated sections were rinsed in PBS, pH 7.4 for 10 min, after which they were: (1) incubated with anti-AVP (Truus, 18/9/85) 1:800 in 0.5% Triton in PBS overnight at 4 °C; (2) washed in PBS (2 × 10 min); (3) incubated with goat anti-rabbit serum (Betsie) 1:50 in PBS for 30 min; (4) washed in PBS (2 × 10 min); (5) incubated with peroxidase–antiperoxidase (PAP) 1:500 to 1:1000 for 30 min; (6) washed in PBS (2 × 10 min); (7) rinsed in 0.05 M Tris-HCl (Merck), pH 7.6; (8) incubated in 0.05 mg/ml 3,3-diaminobenzidine (Sigma) in 0.05 M Tris-HCl, pH 7.6, 0.01% H<sub>2</sub>O<sub>2</sub> (Merck) for 10 min; (9) washed in aqua dest; (10) dehydrated in ethanol and mounted in Entellan. In the sections stained with anti-vasopressin, the borders of the SCN can be delineated reliably (32) (figure 79.1).

### Morphometry

Area measurements of the vasopressinergic SCN and its cell nuclei were performed unilaterally by means of a digitizer (Calcomp 2000) connected to a VAX 11/780, using a Zeiss microscope equipped with 10× and 40× (PLAN) objectives, respectively, and with 12.5× (PLAN) oculars. In order to describe the shape of this nucleus (32) the rostrocaudal axis, the maximal cross-sectional area covered by vasopressin cells and fibers and the SCN volume were determined. The rostrocaudal axis was determined by staining every 25th section with anti-vasopressin starting from the lamina terminalis and ending at the caudal end of the optic chiasm. The rostral and caudal borders of the SCN were assessed by staining every 10th section in the area, and by determining the sections in which, respectively, the first and the last vasopressin cells were present. The maximal cross-sectional SCN area covered with vasopressin cells in the rostrocaudal series was determined as a separate parameter. The volume of the SCN was determined by integrating all the area mea-

surements (37) of the SCN sections that contained immunocytochemically stained cells.

In addition, the vasopressin and total cell number were determined in the SCN of each subject. The number of vasopressin cells per unit SCN volume was estimated using a discrete "unfolding" procedure (39), which included the modification proposed by Cruz-Orive (9) and a correction for section thickness (6 μm). The total SCN cell number was estimated by counting the profile density per unit area in thionin-counterstained material by means of the same procedure. For this purpose the section with the maximal SCN area was selected and nuclear profiles were determined per subject. The computer programs for these procedures were developed by Dr. R. W. H. Verwer at our Institute (for details, see ref. 32).

The volume and total cell number of the SDN were measured in the same way at the same side of the brain in thionin-stained sections (for details, see ref. 31).

Differences among the groups were tested two-tailed using the Kruskal–Wallis multiple comparisons test statistic (7). Throughout this study values are expressed as medians ± the standard deviation of the median. The critical level for statistical significance was taken to be 5%.

### Results

The SCN volume in homosexual males was 1.73 times larger than in the male subjects of the reference group ( $0.463 \pm 0.066 \text{ mm}^3$  and  $0.267 \pm 0.030 \text{ mm}^3$ , respectively;  $P < 0.01$ ) (figure 79.2A) and contained 2.09 times as many cells ( $97.5 \pm 14.6 \times 10^3$  and  $46.7 \pm 7.6 \times 10^3$ , respectively;  $P < 0.001$ ) (figure 79.2B). Similar differences between these two groups were found for the number of vasopressin neurons ( $15.0 \pm 2.1 \times 10^3$  and  $7.9 \pm 1.3 \times 10^3$ , respectively;  $P < 0.01$ ) (figure 79.2C). In addition, the rostrocaudal axis of the SCN was longer in homosexual males than in the male subjects of the reference group ( $2.37 \pm 0.35 \text{ mm}$  and  $1.47 \pm 0.42 \text{ mm}$ , respectively;  $P < 0.02$ ), whereas no such differences were found in the maximal cross-sectional area of the nucleus. In other words, the enlarged volume of the SCN in male homosexuals is mainly due to an extension of this nucleus in rostrocaudal direction, as a result of which homosexuals have a more elongated SCN than heterosexuals. Because the SCN in heterosexual patients who died from AIDS, was not significantly different in volume or total cell number from that of the reference group, an enlarged SCN seems not to be related to the terminal course of illness or to AIDS (table 79.1) but rather to homosexuality. Since the number of vasopressin neurons was smaller in the heterosexual AIDS group than in the ref-

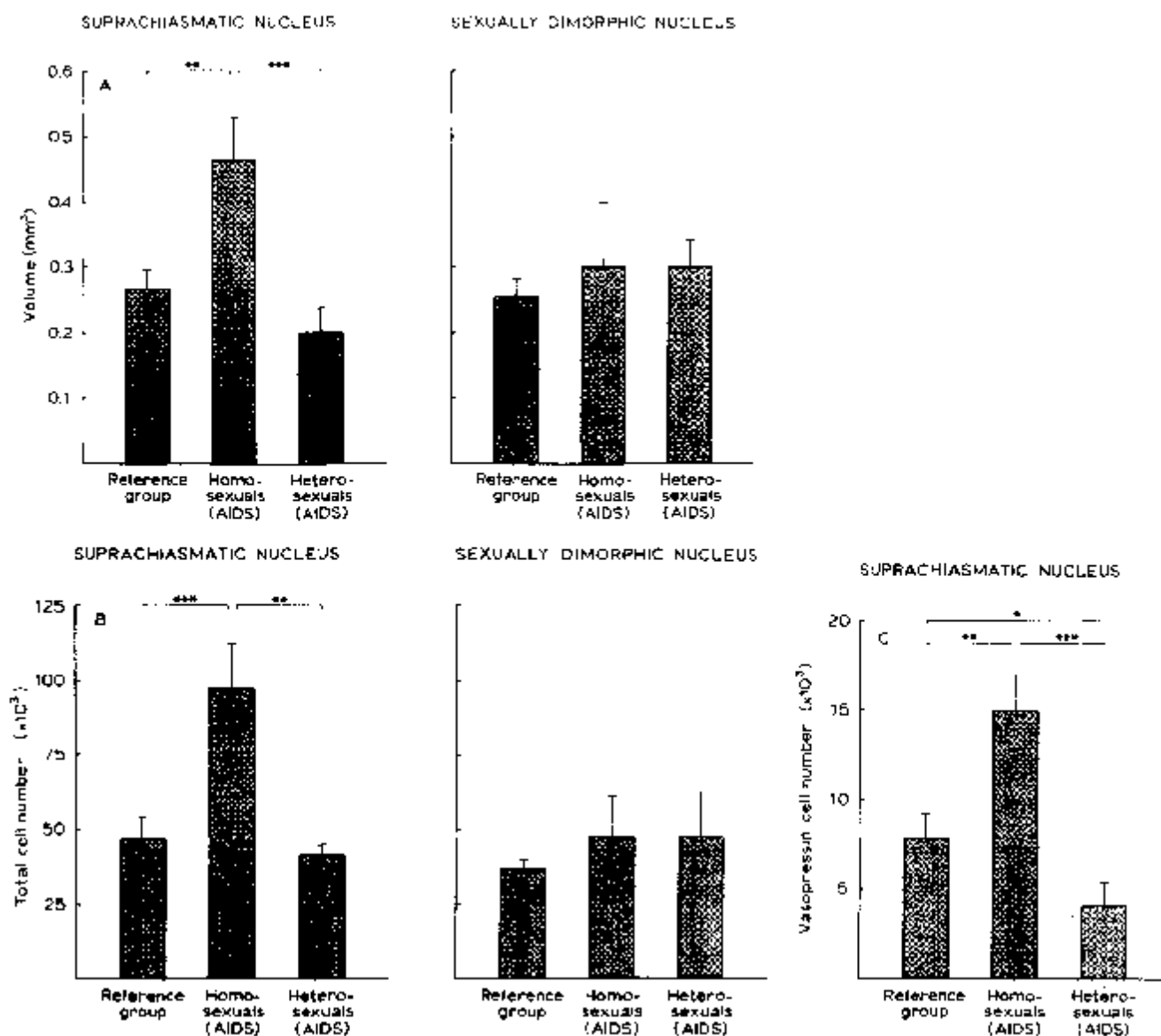


Figure 79.2

*A:* volume of the human SCN and SDN as measured in 3 groups of adult subjects: (1) a male reference group ( $n = 18$ ); (2) male homosexuals who died from AIDS ( $n = 10$ ); and (3) heterosexuals who died from AIDS ( $n = 6$ ; 4 males and 2 females). The values indicate medians and the standard deviation of the median (23). The differences in the volume of the SCN between homosexuals and the subjects from both other groups, are statistically significant. (Kruskal-Wallis multiple comparison-test,  $*P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ ). Note that none of the parameters measured in the SDN (figure 79.2*A,B*) showed significant differences among the 3 groups ( $P$  always  $> 0.4$ ). *B:* total number of cells in the human SCN and SDN. The SCN in homosexual men contains 2.1 times as many cells as in the reference group of male subjects and 2.4 times as many cells as the SCN in heterosexual AIDS patients. *C:* the number of vasopressin neurons in the human SCN (the human SDN does not contain vasopressin-producing cells (22, 31, 34). The SCN in homosexual men contains, on average, 1.9 times as many vasopressin (VP) producing neurons as the reference group of male subjects and 3.6 times as many VP neurons as the SCN in heterosexual AIDS patients. Notice that the SCN of heterosexual individuals who died from AIDS, contains less vasopressin cells than the subjects from the reference group.

erence group ( $P < 0.05$ ) (figure 79.2C), while the total cell number was unaltered (figure 79.2B), AIDS seems to be accompanied by a reduction in the number of neurons expressing vasopressin. This might imply that the number of vasopressin neurons in homosexual men without AIDS may even be higher than observed in figure 79.2. Cell numbers in the SDN of the reference group, the male homosexuals and the heterosexual subjects did not differ significantly implying some degree of selectivity for the SCN enlargement in the hypothalamus of homosexual men.

## Discussion

The prominent theory on sexual orientation, i.e. that it develops as a result of an interaction between the developing brain and sex hormones (13, 15) does not seem to be supported by our data on the SDN. Maternal stress (14) or chemicals (34) are thought to influence the process of sexual differentiation of the brain. According to Dörner's hypothesis, male homosexuals would have a female differentiation of the hypothalamus. This hypothesis was, however, so far based solely on indirect evidence, i.e., the existence of a positive feedback on luteinizing hormone secretion in some homosexual men following injection of estrogens (13, 15). However, according to Gooren (16, 17), this phenomenon is probably related to changes in testicular function rather than to sexual orientation and in his studies could be demonstrated as often in homosexual as in heterosexual men. Dörner's hypothesis became directly testable when we found that the SDN of the preoptic area of the human hypothalamus contains twice as many cells in men as in women (22, 31, 34). The SDN was first described in the rat (18) where it appears to be involved in male sexual behavior (2, 12, 36). Neither the SDN volume nor the cell number in the hypothalamus of homosexual men who died from AIDS, however, differed from that of the male reference group in the same age range (34). The present data confirm and extend this observation with a heterosexual control group of subjects also suffering from AIDS. The fact that no difference in SDN cell number was observed between homo- and heterosexual men who died from AIDS ( $P = 0.50$ ) refutes the most global formulation of Dörner's hypothesis that male homosexuals have "a female brain."

The present data revealed that the volume of the SCN in homosexual men is 1.7 times as large as that of a reference group. Since the SCN in the former group also contains 2.1 times as many cells, this difference cannot be attributed to differences in shrinkage. The difference in SCN cell number in relation to sexual orientation can, however, not be directly related to

sexual differentiation of the brain since no differences in SCN volume or cell number were found between males and females (21, 32). The possibility cannot be excluded, yet, that sex hormone levels during brain development do play some part in this phenomenon (see below).

The association between a large SCN (and, in particular, an increase in the number of neurons) and male homosexuality raises a number of questions about the way it might have arisen. It appears very unlikely that homosexual behavior would increase the neuronal number in any brain structure. The nerve cells of the SCN are postmitotic from a few years of age onwards, if not earlier (35). An increase in stainability of vasopressin neurons due to homosexual behavior is also unlikely, since the vasopressin cell densities do not differ among the 3 groups ( $P > 0.2$ ). Although such a functional interpretation of the data cannot be totally excluded, the development of SCN cell numbers suggests that the explanation for the large SCN in homosexual men most likely may be found in early brain development. At birth, the SCN contains only 13–20% of the adult number of vasopressin and total cells, but in the postnatal period development is rapid. Cell counts reach a peak around 13–16 months after birth (35) and are then of a similar magnitude as in adult homosexual men. In the reference group, the vasopressin and total cell numbers subsequently decline to the adult value of about 35% of the peak values. In homosexual men, therefore, this programmed postnatal cell death in the SCN seems to be limited. The observation that a similarly enlarged SCN was present in a woman with Prader-Willi syndrome (33), a congenital luteinizing hormone-releasing hormone deficiency in which sex hormone levels are very low (4), suggests that the interaction with sex hormones in some stage of development might be essential for the programmed SCN cell death. The possibility of sex hormones playing some role in SCN development is reinforced by an observation of Södersten et al. (30). They showed that the amplitude of the daily rhythm in sexual behavior, for which the SCN is responsible, is enhanced by anti-estrogen treatment of the neonatal animal. This observation and the large SCN in Prader-Willi syndrome (33) suggests that a larger SCN, as reported here for homosexual men, may relate to a difference in the interaction with sex hormones during development. This possibility should be tested in animal experiments.

Dementia as a result of AIDS (26) does not seem to affect the size and cell number of the SCN in a significant manner, as can be concluded from comparing a group of 4 demented homosexual patients who died from AIDS, with the group of 10 non-demented homosexual AIDS patients from the present study. How-

ever, because of the dementia, these 4 cases were not included in the present study.

One might argue that the present finding of an enlarged SCN in male homosexuals who died from AIDS, holds only for a particular subset of homosexual men, i.e., those with a high number of frequently changing sexual partners with whom anal receptive sexual techniques were performed (10, 38). This possibility, i.e. that an enlarged SCN may be related to, e.g., the level of sexual activity rather than to homosexuality certainly warrants further study. Experiments in rats, however, have shown a close correlation between sexual activity and SDN size (2). Our observation that the size of the SDN in homosexual men did not differ from that of the male reference group nor from that of the heterosexual men that died from AIDS, does not support this possibility.

An alternative explanation for the enlarged SCN found in male homosexuals is that it might be related to hypogonadism in adulthood, as has been found in AIDS patients (8). The observation that the SCN in heterosexual male AIDS patients is not enlarged seems to exclude this possible explanation, but homosexual men who did not die of AIDS should certainly be studied in the future. In this respect, it is interesting that we observed an enlarged SCN in two male-to-female transsexuals who did not suffer from AIDS (33).

The conclusion of the present paper is that the SDN data do not support the global hypothesis that homosexual men have a "female brain" (13, 15). An association was found, however, between sexual orientation in men and SCN size, from which the functional implications are momentarily not clear. However, various observations in animals suggest that the SCN, apart from being the biological clock, may be involved in reproductive processes (30, 33). The SCN is also activated around puberty (1). In addition, lesions of the SCN area in the female rat attenuated positive feedback response of gonadotropic hormones to estrogens (20, 40). The relationship between a large SCN and homosexuality is, of course, not necessarily a causal one. Animal experimental research has to reveal whether the SCN is causally implicated in sexual orientation, or whether SCN size and sexual preference are influenced by a common factor during development.

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Sexual orientation—specifically, the direction of sexual feelings or behavior toward members of one's own or the opposite sex—has traditionally been studied at the level of psychology, anthropology, or ethics (1). Although efforts have been made to establish the biological basis of sexual orientation, for example, by the application of cytogenetic, endocrinological, or neuro-anatomical methods, these efforts have largely failed to establish any consistent differences between homosexual and heterosexual individuals (2, 3).

A likely biological substrate for sexual orientation is the brain region involved in the regulation of sexual behavior. In nonhuman primates, the medial zone of the anterior hypothalamus has been implicated in the generation of male-typical sexual behavior (4). Lesions in this region in male monkeys impair heterosexual behavior without eliminating sexual drive (5). In a morphometric study of the comparable region of the human hypothalamus (from men and women of unknown sexual orientation), two small groups of neurons (INAH 2 and 3) were reported to be significantly larger in men than women (6). Thus, these two nuclei could be involved in the generation of male-typical sexual behavior.

I tested the idea that one or both of these nuclei exhibit a size dimorphism, not with sex, but with sexual orientation. Specifically, I hypothesized that INAH 2 or INAH 3 is large in individuals sexually oriented toward women (heterosexual men and homosexual women) and small in individuals sexually oriented toward men (heterosexual women and homosexual men). Because tissue from homosexual women could not be obtained, however, only that part of the hypothesis relating to sexual orientation in men could be tested.

Brain tissue was obtained from 41 subjects at routine autopsies of persons who died at seven metropolitan hospitals in New York and California. Nineteen subjects were homosexual men who died of complications of acquired immunodeficiency syndrome (AIDS) (one bisexual man was included in this group). Sixteen subjects were presumed (7) heterosexual men: six of these subjects died of AIDS and ten of other causes (8). Six

subjects were presumed heterosexual women. One of these women died of AIDS and five of other causes (8). The mean age of the homosexual men was 38.2 years (range, 26 to 53 years), that of the heterosexual men was 42.8 years (range, 33 to 59 years), and that of the women was 41.2 years (range, 29 to 59 years). The subjects were younger and closer in age than those studied in previous investigations: tissue was not taken from elderly heterosexual men or women so that an approximate age-match would be preserved with the homosexual men, who were predominantly young or middle-aged adults (9).

The brains were fixed by immersion for 1 to 2 weeks in 10 or 20% buffered formalin and then sliced by hand at a thickness of about 1 cm in, or close to the coronal plane. Tissue blocks containing the anterior hypothalamus were dissected from these slices and stored for 1 to 8 weeks in 10% buffered formalin. These blocks were then given code numbers; all subsequent processing and morphometric analysis was done without knowledge of the subject group to which each block belonged. The blocks were infiltrated with 30% sucrose and frozen-sectioned at a thickness of 52  $\mu$ m in planes parallel to the original slices. The sections were mounted serially on slides, dried, defatted in xylene, stained with 1% thionin in acetate buffer (15 to 30 min), and differentiated with 5% rosin in 95% alcohol (4 to 10 min). With the aid of a compound microscope equipped with a camera lucida attachment, the outlines of four nuclei (INAH 1, 2, 3, and 4) were traced in every section at a linear magnification of  $\times 83$ . These four nuclei included the two nuclei reported by Allen et al. (6) to be sexually dimorphic and two other nuclei (INAH 1 and 4) for which no sex differences were found (6). The criteria described in (6) were followed in identifying and delineating the nuclei (figure 80.1). The outline of each nucleus was drawn as the shortest line that included every cell of the type characteristic for that nucleus, regardless of cell density. In 15 cases the nuclei in both left and right hypothalami were traced. In 12 cases only the left hypothalamus was studied, and in 14 cases only the right. The areas of the traced outlines were determined with a digitizing

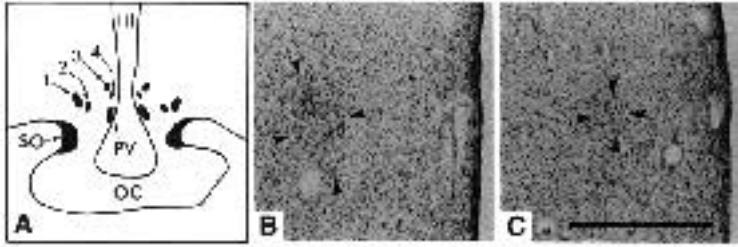


Figure 80.1

(A) Semidiagrammatic coronal section through the human hypothalamus at the level of the optic chiasm (OC). The four cell groups studied (INAH 1, 2, 3, and 4) are indicated by the corresponding numerals. All four nuclei are not generally visible in the same coronal section: INAH 1 lies most anteriorly and INAH 4 most posteriorly. Supraoptic nucleus, SO; paraventricular nucleus, PV; and third ventricle, III. (B) Micrograph of INAH 3 from the left hypothalamus of a heterosexual male. The third ventricle is at the right of the figure. Arrowheads outline INAH 3. (C) Section from a homosexual male comparable to that in (B). INAH 3 is poorly recognizable as a distinct nucleus, but scattered cells similar to those constituting the nucleus in the heterosexual men were found within the area indicated by the arrowheads. The illustrated sections are near the middle of the anteroposterior extent of the nucleus in each case. The scale bar (1 mm) applies to (B) and (C).

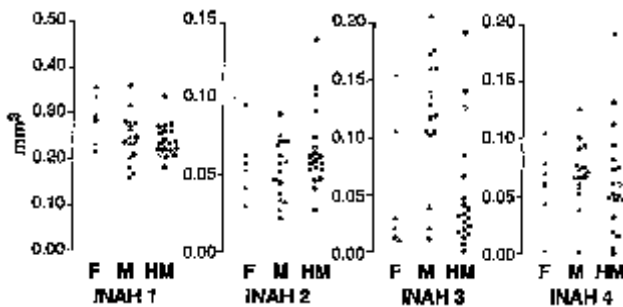


Figure 80.2

Volumes of the four hypothalamic nuclei studied (INAH 1, 2, 3, and 4) for the three subject groups: females (F), presumed heterosexual males (M), and homosexual males (HM). Individuals who died of complications of AIDS, ●; individuals who died of causes other than AIDS, ▲; and an individual who was a bisexual male and died of AIDS, ○. For statistical purposes this bisexual individual was included with the homosexual men.

tablet, and the volume of each nucleus was calculated as the summed area of the serial outlines multiplied by the section thickness.

In the 15 cases where both left and right sides were studied, no significant interhemispheric differences were found for any of the four nuclei. Therefore, in further analysis, the mean of the two sides was used, and the cases where only one side was available were analyzed without regard to the side of origin.

One-way analysis of variance (ANOVA) was used to look for significant differences between subject groups (figure 80.2). No differences were found for INAH 1, 2, or 4. These results for INAH 1 and 4 are consistent with those of Allen et al. (6, 10). However, INAH 2 was reported to be about twofold larger in men than women (6). The failure to replicate that finding may have to do with the relatively young age of the subjects in the present study; as noted in (6), no sex difference was apparent when women of reproductive age were compared with men of similar ages. Thus INAH 2 is

not dimorphic either with sex or with sexual orientation, at least within the age range studied.

INAH 3 did exhibit dimorphism. One-way ANOVA showed that the three sample groups (from women, heterosexual men, and homosexual men) were unlikely to have come from the same population ( $P = 0.0014$ ). Consistent with the hypothesis outlined above, the volume of this nucleus was more than twice as large in the heterosexual men ( $0.12 \pm 0.01 \text{ mm}^3$ , mean  $\pm$  SEM) as in the homosexual men ( $0.051 \pm 0.01 \text{ mm}^3$ ). Because of uncertainty about the nature of the underlying distribution, the significance of this difference was evaluated by a Monte Carlo procedure (11); this showed the difference to be highly significant ( $P = 0.001$ ). The difference was still significant when the homosexual men were compared with only the six heterosexual men who died of complications of AIDS ( $P = 0.028$ ). There was a similar difference between the heterosexual men and the women (mean  $0.056 \pm 0.02 \text{ mm}^3$ ;  $P = 0.019$ ), replicating the observations in (6). There was no significant difference in the volume of INAH 3 between the heterosexual men who died of AIDS and those who died of other causes or between the homosexual men and the women. These data support the hypothesis that INAH 3 is dimorphic not with sex but with sexual orientation, at least in men (12).

INAH 3 is situated about 1 mm lateral to the wall of the third ventricle, and about 1 to 2 mm dorsal to the anterior tip of the paraventricular nucleus. It is spherical or ellipsoidal and contains relatively large, densely staining, polygonal neurons (figure 80.1B). The borders of the nucleus are not well demarcated; hence a blind procedure was used to reduce bias effects. In most of the homosexual men (and most of the women), the nucleus was represented only by scattered cells (figure 80.1C). Because of the difficulty in precisely defining the neurons belonging to INAH 3, however, no attempt was made to measure cell number or density.

Brain tissue from individuals known to be homosexual has only become available as a result of the AIDS epidemic. Nevertheless, the use of this tissue source raises several problems. First, it does not provide tissue from homosexual women because this group has not been affected by the epidemic to any great extent. Thus, the prediction that INAH 3 is larger in homosexual than in heterosexual women remains untested. Second, there is the possibility that the small size of INAH 3 in the homosexual men is the result of AIDS or its complications and is not related to the men's sexual orientation. This does not seem to be the case because (i) the size difference in INAH 3 was apparent even when comparing the homosexual men with heterosexual AIDS patients, (ii) there was no effect of AIDS on the volumes of the three other nuclei examined (INAH 1, 2, and 4), and (iii) in the entire sample of AIDS patients, there was no correlation between the volume of INAH 3 and the length of survival from the time of diagnosis. Nevertheless, until tissue from homosexual men dying of other causes becomes available, the possibility that the small size of INAH 3 in these men reflects a disease effect that is peculiar to homosexual AIDS patients cannot be rigorously excluded.

A third problem is the possibility that AIDS patients constitute an unrepresentative subset of gay men, characterized, for example, by a tendency to engage in sexual relations with large numbers of different partners or by a strong preference for the receptive role in anal intercourse [both of which are major risk factors for acquiring human immunodeficiency virus (HIV) infection (13)]. Sexual activity with large numbers of partners is (or was until recently) common among gay men, however, and therefore does not define an unrepresentative minority (14). In addition, the majority of homosexual men who acquired HIV infection during the Multicenter AIDS Cohort Study (15) reported that they took both the insertive and the receptive role in anal intercourse, and the same is likely to be true of the homosexual subjects in my study. Nevertheless, the use of postmortem material, with the consequent impossibility of obtaining detailed information about the sexuality of the subjects, limits the ability to make correlations between brain structure and the diversity of sexual behavior that undoubtedly exists within the homosexual and the heterosexual populations.

The existence of "exceptions" in the present sample (that is, presumed heterosexual men with small INAH 3 nuclei, and homosexual men with large ones) hints at the possibility that sexual orientation, although an important variable, may not be the sole determinant of INAH 3 size. It is also possible, however, that these exceptions are due to technical shortcomings or to misassignment of subjects to their subject groups.

The discovery that a nucleus differs in size between heterosexual and homosexual men illustrates that sexual orientation in humans is amenable to study at the biological level, and this discovery opens the door to studies of neurotransmitters or receptors that might be involved in regulating this aspect of personality. Further interpretation of the results of this study must be considered speculative. In particular, the results do not allow one to decide if the size of INAH 3 in an individual is the cause or consequence of that individual's sexual orientation, or if the size of INAH 3 and sexual orientation covary under the influence of some third, unidentified variable. In rats, however, the sexual dimorphism of the apparently comparable hypothalamic nucleus, the sexually dimorphic nucleus of the preoptic area (SDN-POA) (16), arises as a consequence of the dependence of its constituent neurons on circulating androgen during a perinatal sensitive period (17). After this period, even extreme interventions, such as castration, have little effect on the size of the nucleus. Furthermore, even among normal male rats there is a variability in the size of SDN-POA that is strongly correlated with the amount of male-typical sexual behavior shown by the animals (18). Although the validity of the comparison between species is uncertain, it seems more likely that in humans, too, the size of INAH 3 is established early in life and later influences sexual behavior than that the reverse is true. In this connection it would be of interest to establish when the neurons composing INAH 3 are generated and when they differentiate into a dimorphic nucleus.

## References and Notes

1. For examples of the variety of approaches to the topic, see S. Freud [*Three Essays on the Theory of Sexuality*, in *Collected Works of Freud*, J. Strachey, Ed. and Transl. (Hogarth, London, 1959), pp. 125–243], C. S. Ford and F. A. Beach [*Patterns of Sexual Behavior* (Ace, New York, 1951)], Vatican Council II [*Declaration on Certain Problems of Sexual Ethics*, in *Vatican Collection*, A. Flannery, Ed. and Transl. (Eerdmans, Grand Rapids, MI, 1982), vol. 2, pp. 486–499], M. Ruse, *J. Homosex.* 6, 5 (1981), and R. C. Friedman [*Male Homosexuality: A Contemporary Psychoanalytic Perspective* (Yale Univ. Press, New Haven, CT, 1988)].
2. M. Pritchard, *J. Ment. Sci.* 108, 616 (1962); H. F. L. Meyer-Bahlburg, *Prog. Brain Res.* 61, 375 (1984); G. Dörner et al., *Arch. Sex. Behav.* 4, 1 (1975); S. E. Hendricks et al., *Psychoneuroendocrinology* 14, 177 (1989); D. F. Swaab and M. A. Hofman, *Dev. Brain Res.* 44, 314 (1988).
3. The suprachiasmatic nucleus (SCN) of the hypothalamus has been reported to be larger in homosexual than in heterosexual men [D. F. Swaab and M. A. Hofman, *Brain Res.* 537, 141 (1990)]. There is little evidence, however, to suggest that SCN is involved in regulation of sexual behavior aside from its circadian rhythmicity [P. Södersten, S. Hansen, B. Srebo, *J. Endocrinol.* 88, 125 (1981)].
4. A. A. Perachio, L. D. Marr, M. Alexander, *Brain Res.* 177, 127 (1979); Y. Oomura, H. Yoshimatsu, S. Aou, *ibid.* 266, 340 (1983).
5. J. C. Slimp et al., *ibid.* 142, 105 (1978).
6. L. S. Allen, M. Hines, J. E. Shryne, R. A. Gorski, *J. Neurosci.* 9, 497 (1989).

7. Two of these subjects (both AIDS patients) had denied homosexual activity. The records of the remaining 14 patients contained no information about their sexual orientation; they are assumed to have been mostly or all heterosexual on the basis of the numerical preponderance of heterosexual men in the population [A. C. Kinsey, W. B. Pomeroy, C. E. Martin, *Sexual Behavior in the Human Male* (Saunders, Philadelphia, 1948)].
8. The causes of death for the ten male subjects who did not die of AIDS were lung carcinoma (two cases), renal failure (two cases), coronary thrombosis, acute lymphocytic leukemia, amyotrophic lateral sclerosis, pancreatic carcinoma, pulmonary embolism, and aspiration pneumonia. For the five female subjects who did not die of AIDS, the causes of death were systemic lupus erythematosus, pancreatic carcinoma, liver failure (two cases), and abdominal sepsis secondary to renal transplantation. All six of the heterosexual male AIDS patients and three of the homosexual men had histories of intravenous drug abuse. Three of the women, two heterosexual men who did not have AIDS, and one homosexual man had histories of chronic alcohol abuse.
9. Criteria for inclusion of subjects in the study were as follows: (i) age 18 to 60, (ii) availability of medical records, (iii) in AIDS patients, statement in the records of at least one AIDS risk group to which the patient belonged (homosexual, intravenous drug abuser, or recipient of blood transfusions), (iv) no evidence of pathological changes in the hypothalamus, and (v) no damage to the INAH nuclei during removal of the brain or transection of these nuclei in the initial slicing of the brain. Fourteen specimens (over and above the 41 used in the study) were rejected for one of these reasons; in all cases the decision to reject was made before decoding.
10. INAH 1 is the same as the nucleus named the "sexually dimorphic nucleus" and reported to be larger in men than women [D. F. Swaab and E. Fliers, *Science* 228, 1112 (1985)]. My results support the contention by Allen et al. (6) that this nucleus is not dimorphic.
11. The ratio of the mean INAH 3 volumes for the heterosexual and homosexual male groups was calculated. The INAH 3 volume values were then randomly reassigned to the subjects, and the ratio of means was recalculated. The procedure was repeated 1000 times, and the ordinal position of the actual ratio in the set of shuffled ratios was used as a measure of the probability that the actual difference between groups arose by chance. Only one of the shuffled ratios was larger than the actual ratio, giving a probability of 0.001.
12. Application of ANOVA or correlation measures failed to identify any confounding effects of age, race, brain weight, hospital of origin, length of time between death and autopsy, nature of fixative (10 or 20% formalin), duration of fixation, or, in the AIDS patients, duration of survival after diagnosis, occurrence of particular complications, or the nature of the complication or complications that caused death. There were no significant positive or negative correlations between the volumes of the four individual nuclei across the entire sample, suggesting that there were no unidentified common-mode effects such as might be caused by variations in tissue shrinkage. The mean brain weight for the women ( $1256 \pm 41$  g) was smaller than that for either the heterosexual ( $1364 \pm 46$  g) or the homosexual ( $1392 \pm 32$  g) men, but normalizing the data for brain weight had no effect on the results. There was no correlation between subject age and the volume of any of the four nuclei, whether for the whole sample or for any subject group; this finding does not necessarily conflict with the report in (6) of age effects in INAH 1, and possibly INAH 2, because in (6) a much wider range of ages was examined than was used in the present study.
13. J. S. Chmiel et al., *Am. J. Epidemiol.* 126, 568 (1987); W. Winkenstein, Jr., et al., *J. Am. Med. Assoc.* 257, 321 (1987).
14. In the largest relevant study [A. P. Bell and M. S. Weinberg, *Homosexualities: A Study of Diversity among Men and Women* (Simon and Schuster, New York, 1978)], nearly half the homosexual male respondents reported having had over 500 sexual partners.
15. R. Detels et al., *J. AIDS* 2, 77 (1989).
16. R. A. Gorski, J. H. Gordon, J. E. Shryne, A. M. Southam, *Brain Res.* 148, 333 (1978).
17. K. D. Döhler et al., *ibid.* 302, 291 (1984); R. E. Dodson, J. E. Shryne, R. A. Gorski, *J. Comp. Neurol.* 275, 623 (1988); G. J. Bloch and R. A. Gorski, *ibid.*, p. 613; R. W. Rhees, J. E. Shryne, R. A. Gorski, *Dev. Brain Res.* 52, 17 (1990).
18. R. H. Anderson, D. E. Fleming, R. W. Rhees, E. Kinghorn, *Brain Res.* 370, 1 (1986).
19. I thank the pathologists who made this study possible by providing access to autopsy tissue; P. Sawchenko, C. Rivier, S. Rivest, G. Torres, G. Carman, D. MacLeod, S. Lockery, and J. Rice for comments and suggestions; and B. Wamsley for assistance with preparation of the manuscript. Supported by a PHS Biomedical Research Support Grant to the Salk Institute.

A fundamental question of human behavior is the relative contribution of genetic, fetal or neonatal hormonal, and postnatal environmental factors to the etiology of sexual orientation. In laboratory animals, considerable attention has focused on observations that levels of gonadal hormones during a critical period of development determine the sexual differentiation of the brain in terms of many sexually dimorphic functions and structures (1). In contrast, little is known regarding sexual differentiation of the human brain. In terms of function, humans exhibit sex differences in reproductive behavior, gonadotropin secretion (2–4), childhood play behavior (5), and cognitive abilities and cerebral lateralization (6–8). In regions of the brain presumably involved in reproductive function, there are reports of sex differences in the suprachiasmatic nucleus (9), the interstitial nuclei of the anterior hypothalamus 1–3 (10, 11), and the bed nucleus of the stria terminalis (12). In regions of the brain not directly related to reproductive function sex differences occur in cerebral asymmetry (13), in the shape of the corpus callosum (CC) (14, 15), and in the midsagittal area of the massa intermedia and anterior commissure (AC) (16).

Several sexually dimorphic functions and structures reportedly differ in homosexual people. Studies indicate that homosexual men exhibit a luteinizing hormone surge that is intermediate between heterosexual men and women (2, 3), although others have failed to confirm this (4). Morphological differences have been identified in homosexual men in nuclei of the brain that are in regions that influence reproductive physiology and behavior: the volume of the suprachiasmatic nucleus may be larger than in heterosexual men and women (17); and the interstitial nucleus of the anterior hypothalamus 3, which we found to be larger in men than in women (11), is smaller in homosexual men than heterosexual men (18). Homosexual males also exhibit differences in characteristics not directly related to reproductive function: there is an increase in the frequency of left-handedness (19–21), childhood play behavior more often resembles that of girls (22), and scores on tests of visuospatial and verbal abilities (23–26) as well as cerebral lateralization (27) resemble those

of heterosexual women more than those of heterosexual men. However, others using different criteria have not found a correlation between sexual orientation and handedness (28) or cognitive abilities (29). We hypothesized that in humans, as in laboratory animals, there is a correlation in the direction of sexual differentiation within a given individual between sexually dimorphic functional characteristics, including sexual orientation, and structural sex differences in the brain that we have identified (11, 12, 16). We now report that a sexually dimorphic structure *not* directly related to reproductive function, the AC, in homosexual men more closely resembles that of heterosexual women than that of heterosexual men in terms of area at the midsagittal plane.

Tissue from presumed homosexual women was not examined in this study because AIDS, which was present in most of the homosexual male subjects, has not specifically affected homosexual women. Therefore, sexual orientation is generally not noted in hospital medical records of women. In contrast, sexual orientation is generally specified in medical autopsy records of all men with AIDS or other diseases that can be either sexually transmitted or result from immunodeficiency.

## Methods

The brains used in this study, which were obtained from three Southern California hospitals between 1983 and 1991, had been removed within 24 hr postmortem and placed directly into acetate-buffered 10% formalin. The present investigators collected 256 samples of brain tissue containing the midsagittal region of the AC from brains that appeared unaffected by neuropathology during routine autopsy. Subsequent to histological analysis of brain samples, medical records were examined for results from histoneuropathology, neurological disorders, cause of death, age, gender, and sexual orientation. Subjects were eliminated from this study when medical records indicated histoneuropathology or disorder(s) potentially influencing the AC (e.g., demyelinating disease;  $n = 59$ ), bisexual orientation ( $n = 1$ ), or AIDS when all risk factors for the disease

**Table 81.1**

Area of AC at midsagittal plane of the brain, age, brain weight (BW), and area/BW for homosexual men, heterosexual men, heterosexual women, and homosexual men without the two subjects with markedly larger ACs

Group	<i>n</i>	Area, mm <sup>2</sup>	Age, yr	BW, g	Area/BW, (mm <sup>2</sup> /g) × 10 <sup>3</sup>
Homosexual men	30	14.20 ± 0.6* <sup>†</sup>	36.5 ± 1.4	1449 ± 22 <sup>‡</sup>	10.1 ± 0.5 <sup>†</sup>
Heterosexual men	30	10.61 ± 0.5 <sup>†a</sup>	38.7 ± 1.5	1439 ± 20 <sup>§</sup>	7.4 ± 0.4 <sup>†  c</sup>
Heterosexual women	30	12.03 ± 0.5*	39.7 ± 1.8	1275 ± 27 <sup>§b</sup>	9.5 ± 0.4 <sup>  </sup>
Homosexual men-2	28	13.44 ± 0.4 <sup>a</sup>	36.7 ± 1.4	1456 ± 22 <sup>b</sup>	9.5 ± 0.4 <sup>c</sup>

Data are mean scores ± SEMs. Symbol superscripts indicate comparisons between all groups; letter superscripts indicate comparisons between heterosexual men, heterosexual women, and homosexual men, excluding the two homosexual subjects with a markedly larger AC (homosexual men-2). Values with identical superscripts are significantly different at the indicated *P* level (Bonferroni *t* procedure): \*, *P* = 0.018; †, ‡, §, and <sup>b</sup>, *P* < 0.0001; †, *P* = 0.0002; ||, *P* = 0.003; <sup>a</sup>, *P* = 0.0002; <sup>c</sup>, *P* = 0.0007.

were denied by the patient (*n* = 3). Male and female subjects were classified as heterosexual when medical records did not indicate homosexual orientation. This procedure generated 34 homosexual men, 84 heterosexual women, and 75 heterosexual men. Before the evaluation of AC area and from the identification codes that included age, sex, and sexual orientation, the subjects were age-matched as closely as possible to obtain 30 triplets—each including a homosexual man, heterosexual man, and heterosexual woman (unmatched subjects were not further evaluated).

Without knowledge of gender or sexual orientation, the blocks of tissue containing the AC were cut by hand at the midsagittal plane, and this surface was placed against a glass adjacent to a ruler at the same plane, photographed, and made into slides. These images were projected onto white paper and traced twice at different times. The area of each outline was determined by using a Bioquant Hipad digitizer adjusted to correct for magnification (Bioquant IBM program version 2.1; R & M Biometrics, Nashville, TN), and the two measurements of each AC were averaged. In an attempt to verify that individuals who died of AIDS did not have neuropathology known to increase gross neural structure—namely, edema and vacuolization, seven ACs from homosexual males who died of AIDS and seven ACs from heterosexual subjects who did not die of AIDS were randomly selected, sectioned at 60 μm, stained with thionin, and examined under a light microscope by a neuropathologist.

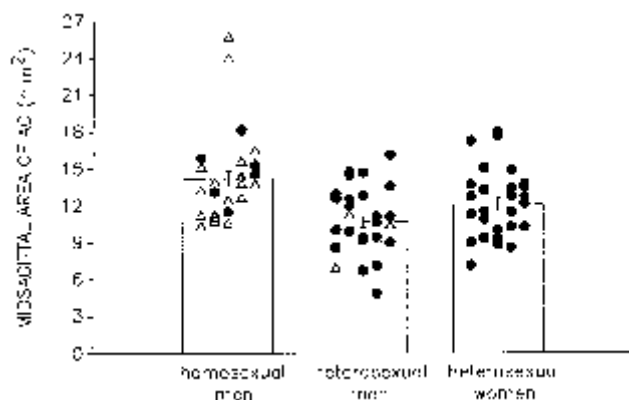
One-way analyses of variance (ANOVA) were performed between the three groups for age, area of AC, brain weight, area of AC divided by brain weight, postmortem period before autopsy, and period between autopsy and photography. To ascertain that our results were not from the two largest ACs of homosexual males, ANOVA were performed excluding these two samples. Similarly, ANOVA were used to compare homosexual men with AIDS, homosexual men without AIDS, heterosexual men with AIDS, and heterosexual men without AIDS. Post hoc comparisons with the

Bonferroni *t* procedure were used to determine differences between the individual groups. Due to the large range of variation in the size of the AC, Mann–Whitney comparisons of AC area between the three groups were made. Pearson's correlation coefficient was used in all subjects and in the three groups individually to test for correlations between the two tracings and between the following parameters: the area of the AC, age, brain weight, postmortem period before autopsy, and period between autopsy and photography. In an attempt to replicate the original observation of a sex difference in the AC (16), a one-tailed independent *t* test compared age-matched heterosexual men and women.

## Results and Discussion

There was a highly significant correlation between the measurements of the two tracings (*r* = 0.94, *P* < 0.0001) with no more than a 5% difference between the two tracings of a given subject. The subjects ranged in age from 22 to 59; ages did not differ between groups (ANOVA, *P* = 0.45) (table 81.1, figure 81.1). There was a significant difference in the area of the AC between the three groups (ANOVA, *P* = 0.0001); the AC of homosexual men was 18.0% or 2.17 mm<sup>2</sup> larger than that of heterosexual women (Bonferroni *t* procedure, *P* < 0.018; Mann–Whitney test, *P* < 0.0075) and 34.0% or 3.6 mm<sup>2</sup> greater than that of heterosexual men (Bonferroni *t* procedure and Mann–Whitney test, *P* < 0.0001). The AC in females was 13.4% or 1.4 mm<sup>2</sup> larger than that of heterosexual men (Bonferroni *t* procedure and Mann–Whitney test, *P* > 0.05).

When the area of the AC in homosexual men without the two subjects with a relatively large AC is compared with the two heterosexual groups, a significant difference remains in the area of the AC and area adjusted for brain weight between heterosexual men but not heterosexual women (table 81.1). Clearly, further studies are needed to determine whether the two



**Figure 81.1**

Midsagittal area of the AC in homosexual men, heterosexual men, and heterosexual women. Data are means  $\pm$  SEMs. Filled circles represent individual subjects without AIDS, and open triangles represent individual subjects who died of AIDS.

large ACs represent a normal variation in homosexual men and whether the AC is, in fact, larger in homosexual men than heterosexual women. Although the AC size did not correlate significantly with brain weight ( $r = 0.019$ ,  $P = 0.86$ ) and the brain weights of homosexual and heterosexual men were similar, the brains of heterosexual females weighed significantly less than those of homosexual ( $P < 0.0001$ ) and heterosexual ( $P < 0.0001$ ) males. Therefore, we adjusted the size of the AC by dividing by brain weight. In this case, the AC of homosexual men was 36.0% greater than that of heterosexual men ( $P = 0.0002$ ) but only 5.9% greater than that of heterosexual women ( $P > 0.05$ ); the AC of heterosexual females was 28.4% greater than that of heterosexual males ( $P = 0.003$ ). There was no difference between groups in postmortem period before autopsy or period between autopsy and photography. Apart from AIDS, there were no striking differences in terms of cause of death between the heterosexual groups.

Subjects who had AIDS included 24 homosexual males, 6 heterosexual males, and no females. ANOVA between men who did and did not die of AIDS indicated that the AC was significantly larger in homosexual than heterosexual men who died of AIDS ( $P = 0.0116$ ) and in homosexual versus heterosexual men who did not die of AIDS ( $P = 0.04$ ). However, within homosexual and heterosexual male groups, there was no significant difference between men who did and did not die of AIDS (table 81.2).

In examining groups of all subjects and the three groups separately for correlations between the area of the AC, age, brain weight, postmortem period before autopsy, and period between autopsy and photography, the only significant correlations were between age and brain weight with age when all subjects

**Table 81.2**

Area of AC at midsagittal plane of the brain for different groups

Group	<i>n</i>	Area, mm <sup>2</sup>
Homosexual with AIDS	24	14.08 $\pm$ 0.16*†
Homosexual without AIDS	6	14.65 $\pm$ 0.94†‡
Heterosexual with AIDS	6	9.63 $\pm$ 0.62*§
Heterosexual without AIDS	24	10.85 $\pm$ 0.57†§

Values (mean  $\pm$  SEM) with superscripts that are the same indicate statistical comparison (Bonferroni *t* procedure): \*,  $P = 0.0116$ ; †,  $P = 0.04$ ; ‡ and §,  $P > 0.05$ .

( $r = -0.3024$ ,  $P = 0.0038$ ) and heterosexual females ( $r = -0.43$ ,  $P = 0.017$ ) were examined.

In a previous study reporting a sex difference in the area of the AC in 100 age-matched male and female subjects, which were all different from those of this study, the AC was 12% greater in females than in males (16). Similarly, in this study when the areas of the AC in 60 age-matched heterosexual subjects are compared, we find a significant difference (one-tailed independent *t* test,  $P = 0.022$ ), confirming our previous finding of a sex difference in area of the AC.

The accuracy of our measurements was limited by the precision by which a true midsagittal section was obtained and of the tracing of the AC in unstained tissue. In an ongoing study where tissue was sectioned coronally at 60  $\mu$ m on a sliding microtome and stained with thionin, thereby permitting greater precision of measurements, a smaller sample size has actually revealed greater differences between the three groups, confirming the results of this study.

Although a majority of homosexual subjects in this study died of AIDS, there is no reason to believe that this disease resulted in an increase in AC area in these subjects. AIDS-related neuropathologies are predominately associated with neural atrophy. Moreover, neuropathology of structures connected by the AC, which may have not been specifically examined during autopsy, should also result in AC atrophy (30). However, two factors that can increase the size of a structure—local vacuolization and edema—were not apparent in any of the AC samples examined. Clearly, other histological preparations could indicate pathologies of the AC, but these would not be expected to increase the AC size. Furthermore, the AC is larger in homosexual than heterosexual men whether we compare subjects of both groups with or without AIDS (table 81.2). Although individuals with AIDS show a high incidence of testicular dysfunction and lower serum testosterone levels (31, 32), and decreased circulating gonadal hormones result in decreased volumes of some sexually dimorphic nuclei (33–35), to our knowledge no study has examined the influence of circulating gonadal hormones on the morphology of structures other than

sexually dimorphic nuclei. Nonetheless, no subjects in this study had medical records indicating testicular dysfunction. Although medical records were used to determine sexual orientation, heterosexual orientation was only assumed, rather than specified, in men and women who did not die of AIDS, hepatitis, or a disease associated with immunocompromise in young and middle-aged people. Clearly, subjects who were classified as heterosexual may not have been, and the degree of homosexuality or heterosexuality was unknown unless medical records indicated bisexuality. However, erroneous classification of subjects is likely to decrease chances of observing significant differences rather than resulting in apparent differences that do not exist.

The AC of the primate brain is a tract of axons that primarily connects the right and left neocortex of the middle and inferior temporal lobes; fewer connections exist between the rostral superior temporal gyrus, olfactory complex, amygdala, and projections to the caudal superior temporal gyrus (36–41). Although subregions of several of these areas exhibit sexual dimorphism in various species, it is unknown whether sexually dimorphic structures in the human brain send or receive projections through the AC. Moreover, it is unknown whether differences in the area of the AC reflect differences in axon number, myelination, vasculature, connective tissue, or glia. In a small human sample, the area of the AC corresponded to the number of axons and not their density; however, in a study of rhesus monkeys, the midsagittal area of the AC did not correlate with the number of axons (42, 43). Although axonal elimination may occur due to atrophy with advancing age, in humans there is protracted myelination of the AC, at least into adulthood (44). Moreover, the size of the AC varies considerably within studies and the means of different studies vary (16, 42, 43, 45, 46). Age-related changes and considerable variation in AC area demonstrate a need for both age-matching and large-sample sizes.

The AC in monkeys may play a role in inhibiting bilateral formation of engrams, thereby increasing both functional asymmetry and mnemonic storage capacity of the brain by preventing redundancy (47). Monkeys with sectioned ACs could differentiate more accurately between left-right mirror images (48). In humans, the AC mediates the interhemispheric transfer of visual, auditory, and olfactory information (49).

The functional significance of differences in the area of the AC is unknown. However, differences in the connectivity between the cerebral hemispheres have been speculated to underlie sex differences between males and females in terms of cerebral lateralization (50, 51). Handedness, which may be a measure of cerebral lateralization, may correlate with sexual orientation (refs. 19–21; see also ref. 28), the size of regions

of another fiber tract, the CC (51), and asymmetries of the temporal lobe (52). In fact, the region of the CC that varies with hand preference may contain fibers connecting asymmetric and sexually dimorphic regions of the temporal lobe (51). Moreover, there is a correlation between the size of the AC, sexual orientation, and scores on tests of verbal and visuospatial abilities and cerebral lateralization: homosexual men and heterosexual women have larger ACs, higher scores on verbal tests, and lower scores on exams of visuospatial abilities (23–26) and cerebral lateralization (27) than heterosexual men (also see ref. 29). However, handedness, determined by a questionnaire on hand preference for a variety of skills (19–21, 51) is not available in the medical records of these deceased subjects. It is currently unknown whether the AC connects asymmetric regions of the brain or whether its midsagittal area correlates with hand preference.

From studies of anatomical sex differences in the human brain (11–16), those differences that (appear to) relate to reproductive functions in which there is little overlap between men and women also exhibit relatively dramatic sex differences, in comparison to those that (appear to) relate to nonreproductive functions, such as cognition, in which considerable overlap occurs between males and females. Consistent with this observation, interstitial nucleus of the anterior hypothalamus 3, which is located in a region of the brain involved in reproductive function, exhibits more dramatic sex- (11) and sexual orientation-related (18) differences than the AC (16), which connects regions of the brain presumably involved in nonreproductive functions.

It is unknown at what period in life sex- and sexual orientation-related differences in the AC develop. However, in laboratory animals, a majority of neuroanatomical sexual dimorphisms arise during perinatal life, although more subtle changes in sexually dimorphic structures can also occur during adulthood (33–35). Because one cannot experimentally alter the hormonal environment of the developing human, it will be difficult to determine whether sexual differentiation of the human brain is influenced by gonadal hormones and/or is modified by environmental factors during fetal or neonatal life, as occurs in laboratory animals (50). However, knowledge of the time course of the establishment of structural sex differences in the human brain and the study of individuals exposed to atypical hormonal environments may be useful in identifying causal factors of homosexuality.

In vivo imaging techniques such as magnetic resonance imaging, which we used to confirm a sex difference in the shape of the corpus callosum in the living human (15), may be used to examine the relationship between sexually dimorphic brain structures and functions such as sexual orientation in both men

and women, cerebral lateralization, and cognition—thereby including studies of homosexuals of both genders and eliminating problems with disease states and uncertain information regarding sexual orientation. The present report of a correlation between sexual orientation and the midsagittal area of the AC, a structure that is both sexually dimorphic and not believed to be related to reproductive function, when combined with reports of similar correlations with hypothalamic nuclei, clearly argues against the notion that a single brain structure causes or results from a homosexual orientation. Rather, this correlation supports the hypothesis that factors operating early in development differentiate sexually dimorphic structures and functions of the brain in a *global* fashion.

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According to animal studies, the choice of sexual partner is highly influenced by sex-specific pheromone signals, which are processed by male and female mating centers located in the anterior hypothalamus (1–3). A lesion of the respective mating center, as well as impairment of pheromone transduction, may alter the coital approach in a sex-specific way (3, 4).

In a majority of animals, pheromone signals are transferred to the hypothalamus from the vomeronasal organ via the accessory olfactory nerve (5). Because our vomeronasal pit lacks neuronal connections to the brain (5, 6), the occurrence of pheromone transduction has long been questioned in humans. Several recent observations, however, suggest that this type of chemical communication cannot be ruled out. Sex steroid-derived compounds such as 4,16-androstadien-3-one (AND) and, less consistently, *estra-1,3,5(10),16-tetraen-3-ol* (EST) have been reported to induce sex-specific effects on the autonomic nervous system, mood, and context-dependent sexual arousal (7–12). The exact effects of AND and EST vary with the administered dose and experimental design, but, nevertheless, they seem to be sex-differentiated (especially with respect to AND), and thus to differ from the effects of ordinary odors. Studies with positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) have shown that smelling both AND and of EST activates the human brain (13–15), even in nonodorous concentrations (14, 15). AND is a derivative of testosterone and is primarily produced in male sweat (16), whereas EST is an estrogen-resembling steroid that has been detected in the urine of pregnant women (17). Male sweat has recently been reported to alter the pulsative hypothalamic release of luteinizing hormone in females in an ovulation-promoting way (18). Thus, although it is premature to classify AND and EST as pheromones, the data suggest that they may function as chemosignals.

In a previous PET study of regional cerebral blood flow (rCBF) in heterosexual subjects (13), we found that smelling AND and EST caused a sex-differentiated activation of the anterior hypothalamus. In women, AND activated the preoptic area and ventromedial nuclei, whereas, in men, activation by EST involved

an area covering the paraventricular and dorsomedial nuclei. In contrast, when men smelled AND and women EST, activations were found only in amygdala plus piriform cortex, anterior insular cortex, orbitofrontal cortex, and anterior cingulate cortex. These areas are reported to process the signals of common odors (19, 20, 21), and were possibly recruited by the odor components of AND and EST. Our interpretation of this sex-differentiated pattern of activation was that the two steroid compounds may act bimodally, both as pheromones and odors. We proposed the hypothesis that the anterior hypothalamus primarily processed signals from the pheromone-like component of AND and EST, whereas the olfactory brain primarily mediated the signals of their odor component. Depending on the sex of the responder in relation to the specific compound (AND or EST), one pathway dominated, whereas the other was suppressed. This hypothesis was based upon observations of a similar phenomenon in studies of other bimodal odorants (for example, acetone) (22).

In the present study, we investigated the question whether the pattern of activation induced by AND and EST could be related to sexual orientation rather than to the biological sex. We therefore compared the pattern of activation between homosexual men and heterosexual men and women. The activations were induced by smelling AND, EST, and ordinary odors (here denoted as OO). Smelling of odorless air served as the baseline condition. The following issues were addressed in particular: (i) In homosexual men (HoM), is the hypothalamus activated by AND, EST, or both? (ii) Is the pattern of activation in HoM similar to that in heterosexual men (HeM) and that in heterosexual women (HeW), or are entirely different regions involved in HoM? (iii) If there are group differences, are they confined to the pheromone-like compounds, or do they occur also with OO?

## Methods

Thirty-six healthy, unmedicated, right-handed, and HIV-negative HeM, HeW, and HoM (12 in each group), who were osmic for both AND and EST and

had normal MRI of the brain, participated in the study. The groups were matched for age ( $28 \pm 2$ ,  $26 \pm 2$ , and  $33 \pm 7$  yr) and educational level, and differed only with respect to biological sex and sexual orientation. All HeW and HeM scored 0, whereas all HoM scored 6 on the Kinsey heterosexual/homosexual scale (0 = maximally heterosexual, 6 = maximally homosexual) (23). Seven HeW, six HeM, and six HoM had a stable sexual partner. Women were investigated during the second to third week of the menstrual cycle.

Before PET experiments, we tested odor thresholds in each subject with the butanol test (21). Using an identical approach, we also tested odor thresholds to AND and EST. For this purpose, the two compounds were diluted in odorless mineral oil. At about 10 a.m. on the day of scanning, blood samples were drawn from each subject for measurements of the concentrations of the following hormones: dehydroepiandrosterone sulfate, follicle-stimulating hormone, luteinizing hormone, bound and free testosterone, prolactin, and androstenedione.

During PET experiments, all of the stimuli were presented in glass bottles at a distance of 10 mm from the nose. The OO consisted of lavender oil, cedar oil, eugenol, and butanol. The butanol was administered at a concentration of 10%; the other odors were undiluted. All of the odor concentrations were thus suprathresholded. Because, theoretically, dissolving of AND and EST could change their possible pheromone properties (24), the respective compound was presented in crystalline and odorous form (200 mg, Steraloids, Newport, RI) during PET scans. The purity was 98%, as tested repetitively at our doping laboratory (Department of Pharmacology, Karolinska University Hospital).

PET measurements were carried out at the same time of day. Furthermore, the room temperature and air pressure were standardized ( $23^{\circ}\text{C}$ , 997 hPa) (21). The three groups of subjects were investigated over the same time period and by the same experimenters. The experimental protocol has been described in detail elsewhere (13, 19, 21). In summary, it included MRI scans, and PET measurements of rCBF with  $^{15}\text{O}$   $\text{H}_2\text{O}$ . The resolution of the PET scanner was 3.8 mm. Four conditions were used: smelling odorless air (baseline condition, denoted here as AIR), smelling AND, smelling EST, and smelling OO. There were 12 scans per person (three scans per condition, balanced and randomly interleaved). During AND, EST, and AIR scans, the same item was presented four times for 15 s per time, with 5 s in between, in an on-off mode. During the OO scans, separate odors were presented consecutively with an identical scheme. The subjects were instructed to breathe normally. They were informed that they would smell odor or odorless air, without knowing the type or order of items.

Respiratory movements were recorded continuously 2 min before, and during each scan, by using a strain gauge around the lower thorax connected to a graph (Comair, Stockholm). After the PET scans, the participating subjects rated AND, EST, and OO for pleasantness, irritability, intensity, and familiarity, using a 100-mm bipolar visual-analogue scale (21).

### Image Analysis

The individual MRI and PET images were reformatted into a common space (standard brain) and filtered with 10-mm Gaussian kernel as described (13, 19, 21). Significant activations were then determined with statistical parametric mapping statistics (SPM99, Wellcome Foundation, London) (25), by using the contrasts AND-AIR, EST-AIR, and OO-AIR. Three types of analyses were performed.

On the basis of previous findings in heterosexual subjects (13), we first investigated whether the hypothalamus in HoM was activated in congruence with that in HeW or HeM. For this purpose, a region of interest (ROI) analysis was carried out. Because of the difficulty in determining the anatomical boundaries of the anterior hypothalamus, the ROIs were defined from hypothalamic activations by AND in HeW (covering the preoptic plus ventromedial nuclei) and by EST in HeM (covering the dorsomedial plus paraventricular nuclei), generated earlier (13). The rCBF was in all subjects first normalized to the global mean in the brain of 50 ml/min per 100 g. The mean rCBF of the three scans per condition (13) was then calculated in each ROI and subject for AIR, AND, and EST. Differences between HoM, HeW, and HeM on the basis of AND-AIR and EST-AIR in each predetermined ROI were tested with two-way repeated measure ANOVA with subject group as the between factor and the type of steroid as within factor. In case of a significant interaction in a main effect, the results were further explored with appropriate contrast. *P* values were considered significant when  $<0.05$ .

There is a possibility, however, that HoM might have an entirely different pattern of activation in extra-hypothalamic structures. The statistical evaluation therefore relied primarily on explorative and user-independent analysis with statistical parametric mapping (SPM99) (25), using the entire brain as search space. Significant activations were first tested in each separate group with one-group random effect analysis (SPM99 basic model, height threshold at  $P = 0.001$ , corrected  $P < 0.05$ ). Next, we used conjunctional analysis (25, 26) to investigate which activations, if any, were common to the two or more groups. Finally, we tested whether there were any differences between HoM, HeM, and HeW with two-group random effect analysis

**Table 82.1**  
Activations

Region	HoM			HeM			HeW		
	<i>z</i> Level	Size, cm <sup>3</sup>	Coordinates	<i>z</i> Level	Size, cm <sup>3</sup>	Coordinates	<i>z</i> Level	Size, cm <sup>3</sup>	Coordinates
<i>EST-AIR</i>									
Hypothalamus				4.2	0.7	+4, -14, -2			
R amygdala plus piriform cortex	4.4	0.3	+24, 0, -6*				4.4	1.1	+34, 0, -14
L amygdala plus piriform cortex	4.5	1.4	-22, -4, -2				4.3	0.3	-20, -24, -8*
Cingulate							4.0	0.6	-8, +30, +34
<i>AND-AIR</i>									
Hypothalamus	5.5	1.0	+8, -2, -2				5.4	0.8	-6, 0, -12
R amygdala plus piriform plus insular cortex				5.1	1.3	+30, 0, -12			
L amygdala plus piriform plus insular cortex				4.4	1.1	+38, -8, +14			
Cingulate				4.5	0.6	-10, +30, -2*			
<i>OO-AIR</i>									
R amygdala plus piriform plus insular cortex	4.2	0.9	+18, +4, -16	4.6	0.8	+22, +4, -12	4.9	3.2	+24, -8, 0
L amygdala plus piriform plus insular cortex	4.6	3.4	-26, -2, -10	5.8	3.0	-18, +4, -13	4.5	0.9	-38, +2, +6

Activations calculated with one-random effect analysis (SPM99). All the significant clusters, calculated with *T*-threshold at  $P = 0.001$  (a corrected  $P < 0.05$ ), are included. Talairach coordinates indicate local maxima. The OO clusters also covered minor portions of anterior cingulate. R, right; L, left.

\*Italics indicate subsignificant clusters calculated at *T*-threshold at  $P = 0.001$  and with a corrected  $P < 0.1$ . These clusters were included to illustrate that the distributions of activations of the olfactory circuits during smelling of the two steroids were similar in the three groups.

(SPM99, basic models, two-group *t* test; height threshold at  $P = 0.001$ , corrected  $P < 0.05$ ) (25).

To locate the hypothalamic clusters more precisely, the coordinates of Talairach's atlas were translated to those of Schaltenbrant's atlas (27, 28), which visualizes the hypothalamic nuclei in detail.

#### Comparisons with Psychophysical Parameters and Hormone Levels

The mean respiratory amplitude and frequency were first calculated during each prescan and scan period. The percentage difference between the scan and prescan value was then compared among HoM, HeM, and HeW with respect to AIR, AND, EST, and OO by using a two-way ANOVA, factoring for subject group and stimulus type including AIR, as described (13, 19). A two-way ANOVA was used also to test group differences in odor ratings, but the stimuli were AND, EST, and OO, because AIR was perceived as odorless. Finally, the group differences in hormone levels and odor thresholds were tested with separate one-way ANOVAs. The significance level was 0.05 for all comparisons.

#### Results

The hypothesis-based ROI analysis showed that the HoM processed AND congruently with HeW rather than with HeM. As in HeW, in HoM, rCBF increased significantly in the preoptic plus ventromedial ROI during smelling of AND ( $P = 0.03$ ), but not of EST ( $P = 0.05$ ). The AND-induced activation was significant compared with AND-AIR in HeM ( $P = 0.03$ ).

EST also induced an increase in rCBF in HoM, but in the dorsomedial plus paraventricular ROI ( $P = 0.0003$ ). As in HeM, this increase was significant compared with EST-AIR in HeW ( $P = 0.01$ ). No other differences between homosexual and heterosexual subjects were observed.

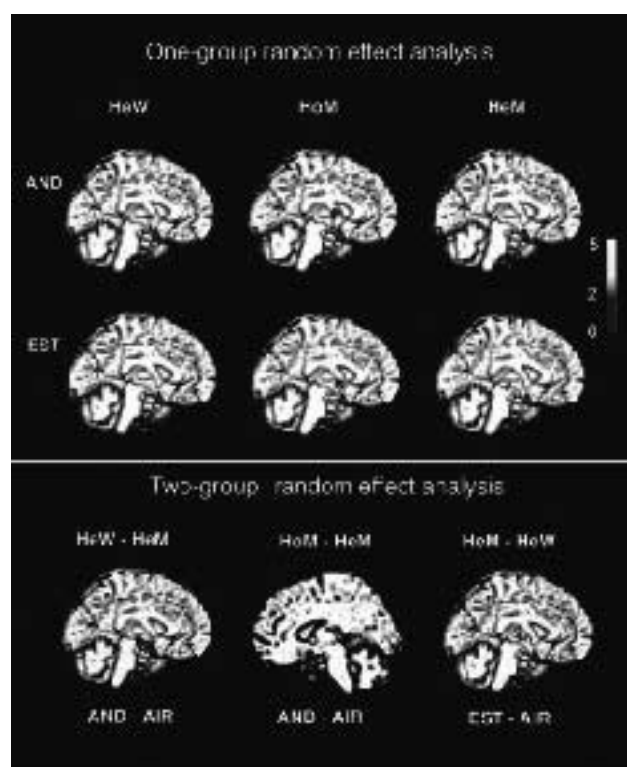
The explorative statistical parametric mapping analysis confirmed the previously reported (13) dissociation of activations by AND and EST, in that HeW showed activation of the anterior hypothalamus with AND, whereas, in HeM, this area was recruited during smelling of EST (tables 82.1 and 82.2 and figure 82.1 *Upper*). As in HeW but not in HeM, in HoM, the anterior hypothalamus was activated with AND. When HoM smelled EST, the left amygdala and piriform cortex were primarily recruited (although with inclusion of a minor portion of the anterior hypothalamus) (table 82.1 and figure 82.1 *Upper*). There was also a subsignificant activation of the right amygdala and piriform cortex. Thus, the HoM showed a pattern of activation that resembled that of HeW rather than of HeM. In contrast to the two steroids, OO yielded similar activations in all three groups. Furthermore, these activations were confined to the olfactory brain (19–21) (table 82.1).

The results (based on one-group random effect analysis) were in accordance with those from conjunctural analysis. HoM shared hypothalamic activation only with HeW, and only when smelling AND (figure 82.2 and table 82.2). HoM thus showed no hypothalamic involvement in common with HeM. In contrast, they shared clusters with the HeM in the amygdala plus piriform plus insular cortex. Conjunctural clusters in

**Table 82.2**  
Conjunctive clusters

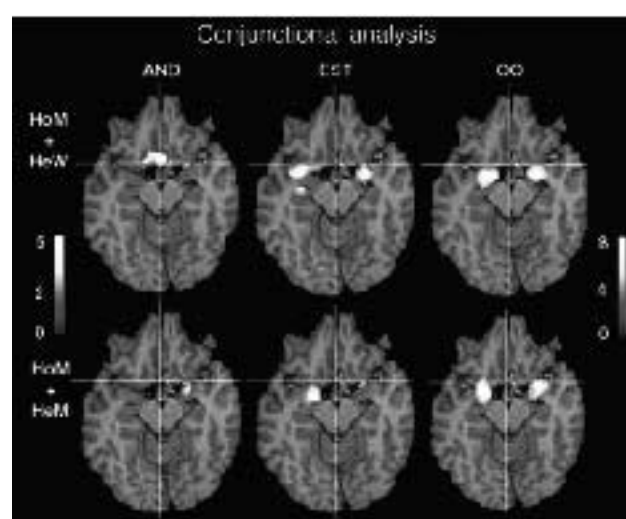
Region	HoM and HeW			HoM and HeM			HeM and HeW		
	<i>z</i> Level	Size, cm <sup>3</sup>	Coordinates	<i>z</i> Level	Size, cm <sup>3</sup>	Coordinates	<i>z</i> Level	Size, cm <sup>3</sup>	Coordinates
<i>EST vs. AIR</i>									
R amygdala plus piriform plus insular cortex	4.9	1.0	+26, -6, -12				4.1	0.4	+34, -10, -8
L amygdala plus piriform plus insular cortex	4.4	1.5	-24, -2, -8	5.1	1.0	-20, +4, -16	4.0	0.8	-24, 0, -12
<i>AND vs. AIR</i>									
Hypothalamus	4.0	0.9	-6, -2, -12						
R amygdala plus piriform cortex				3.7	0.9	+16, +6, -10			
L amygdala plus piriform plus insular cortex							3.6	0.9	-26, +2, -8
<i>OO vs. AIR</i>									
R amygdala plus piriform plus insular plus cortex	6.4	6.4	+22, -2, -12	5.6	5.5	+20, +2, -12	6.3	5.5	+22, 0, -14
L amygdala plus piriform plus insular cortex	6.6	6.6	-20, -2, -10	5.7	5.9	-24, 0, -14	6.4	5.1	-18, -2, -14

Activations calculated with conjunctive analysis (spm99). *T*-threshold at  $P = 0.001$  (corrected  $P < 0.05$ ). R, right; L, left.



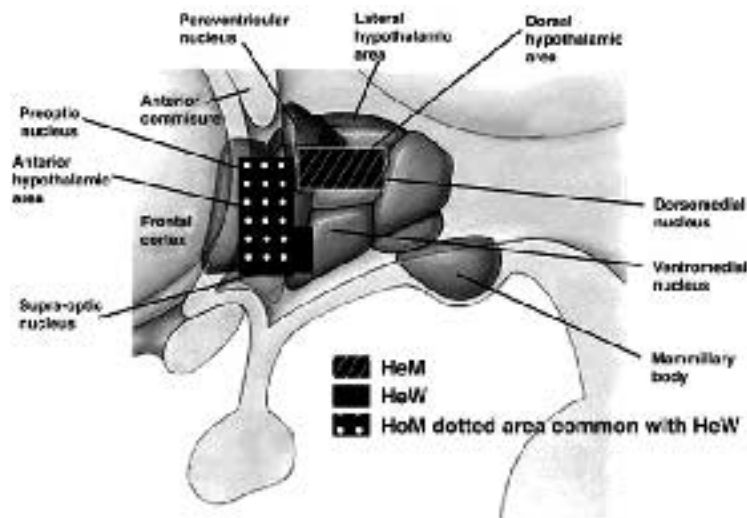
**Figure 82.1**

Illustration of group-specific activations with the putative pheromones. (*Upper*) Cerebral activation during smelling of AND and EST. Clusters of activated regions are superimposed on the standard MRI brain (spm99), midsagittal plane. The inferior portion of the EST cluster in homosexual men is in the amygdala and piriform cortex. (*Lower*) Significant differences between the groups. Shown are the clusters calculated with two-group random effect analysis. The Sokoloff color scale illustrates *z* values reflecting the degree of activation. Only significant activations are shown. Because the same brain section is chosen, the figures do not always illustrate maximal activation for each condition.



**Figure 82.2**

Common activations. Shown are conjunctive clusters in different groups of subjects, superimposed on the standard brain. All images show horizontal level at  $z = -12$  according to Talairach's atlas. The Sokoloff color scale in illustrates *z* values, which are 0.0–5.0 for activations with AND and EST, and 0.0–8.0 for activations with OO. Only significant activations are shown. Because the same brain section is chosen, the figures do not always illustrate maximal activation for each condition. The subject's right side is to the right.



**Figure 82.3**

A schematic presentation of the areas covered by the hypothalamic clusters.

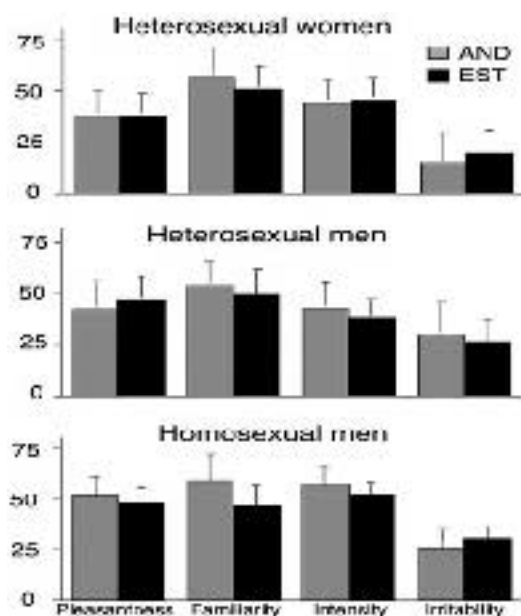
these classical odor-processing regions were observed in all three groups, and independently of the type of odorous stimulus (table 82.2 and figure 82.2).

The group comparisons (two-group random effect analysis) showed that HoM differed only from HeM. This difference consisted in AND-induced activation of the hypothalamus in HoM (Talairach's coordinates in the random effect analysis contrasting HoM–HeM with respect to AND–AIR were +6, +2, +2;  $z = 4.7$ ; size  $0.6 \text{ cm}^3$ , height threshold  $P = 0.001$ ; corrected  $P = 0.05$ ) (figure 82.1). Random effect analysis also showed differences between HeW and HeM. The peak coordinates for this comparison were +3, +2, –13 (yielded by the contrast HeW–HeM with respect to AND–AIR;  $z = 4.2$ , cluster size  $0.8 \text{ cm}^3$ ) and +6, –8, +2 (yielded by the contrast HeM–HeW with respect to EST–AIR;  $z = 4.0$ , cluster size  $0.4$ ; height threshold at  $P = 0.001$ , corrected  $P < 0.05$ ) (figure 82.1). Thus, as reported (13), HeW and HeM differed only regarding the hypothalamic activation by AND and EST (table 82.1).

Given that homosexual behavior can be induced in male ferrets, rats, and mice by damage to the preoptic nucleus (1–3), it was of interest to locate the hypothalamic clusters more precisely. An exact localization was considered relevant despite the 10-mm image filtering, because the clusters were at least 10 mm apart. To justify such an evaluation, we first tested whether the hypothalamic clusters obtained at group level showed a similar localization in each individual of the respective group. Coregistration and repositioning of PET clusters on individual reformatted MRI images revealed similar cluster locations in different subjects, without any systematic shifts between the groups. In all HoM, as in all HeW, the AND cluster incorporated

an area corresponding to the preoptic, ventromedial, and tuberomammillary nuclei (table 82.1). The EST cluster covered the dorsomedial and paraventricular nuclei in HeM (table 82.1). In HoM, the EST cluster showed a local maximum in the amygdala plus piriform cortex, but encompassed a minor portion of the hypothalamus. Because this portion was anterior to the EST-related cluster of HeM, we hypothesized that the possible EST-induced hypothalamic activation in HoM differed from that in HeM. To test this possible difference, a separate post hoc random effect analysis was performed with a reduced search volume, defined with a manually drawn rectangular mask incorporating only the hypothalamus, fornix, and medial amygdalae (Talairach's coordinates:  $x = -20$  to  $+20$ ;  $y = +20$  to  $-40$ ;  $z = -13$  to  $+5$ ). A significant difference was found; as expected, this difference consisted in more pronounced activation in HeM, the maximum corresponding to the location of the dorsomedial nucleus (Talairach's coordinates +8, –10, –2;  $z = 5.4$ , uncorrected  $P = 0.013$ ). The respective hypothalamic clusters are shown schematically in figure 82.3.

These data raised the question whether the direct contrasts between the effects of the two steroids also would differ between homo- and heterosexual subjects. When the entire brain was used as a search space, no clusters were observed for AND–EST and vice versa in any group. However, when applying the rectangular mask described in the previous paragraph, the HeW showed a hypothalamic cluster for AND–EST (+8, –2, –8;  $z$  score 4.1, with a second peak corresponding to Talairach's coordinates of –18, –20, –8; corrected  $P < 0.05$ ); in contrast, a cluster was detected in the amygdala and piriform cortex for EST–AND (+12, +20, –20 and –18, +20, –16;  $z = 4.2$ ). HeM displayed



**Figure 82.4**  
Odor ratings for AND and EST. The vertical axis indicates a visual analogue scale in millimeters (mean  $\pm$  SEM). (Top) Heterosexual women. (Middle) Heterosexual men. (Bottom) Homosexual men. None of the ratings differed between the three groups of subjects.

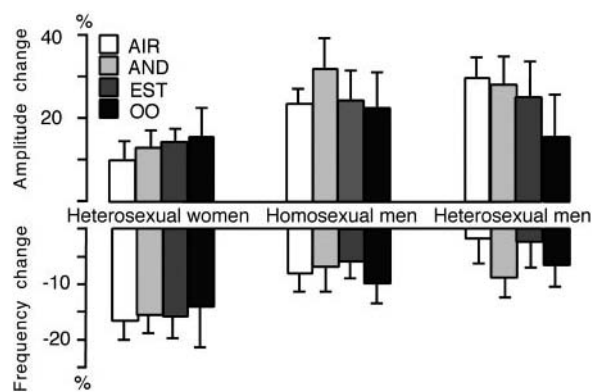
significant EST-AND activation at corrected  $P = 0.08$  with a peak coordinate of  $+4, 0, -2$  ( $z = 4.1$ ); they also displayed AND-EST activations, but in the olfactory circuits: the insula and piriform cortex ( $+30, -2, -2$ ;  $z = 3.8$ ) and the anterior cingulate cortex ( $-10, +32, +6$  and  $+22, +40, +2$ ;  $z = 4$ ). In HoM, clusters were found only at a corrected  $P$  of  $<0.1$ . AND-EST showed a cluster corresponding to the fornix ( $+16, -20, -8$ ;  $z = 4.1$ ), whereas the EST-AND cluster covered the amygdala and piriform cortex ( $-24, -6, -14$ ;  $z = 3.7$ ). No other clusters were found. Together, these data suggest the occurrence of partly overlapping activations induced by AND and EST (see Discussion).

There were no significant group-odor interactions for any of the rating parameters (figure 82.4). Neither did we find any group-stimulus interaction in respiratory amplitude or frequency (figure 82.5).

No group differences were observed either in odor thresholds or plasma concentrations of the tested hormones (tables 82.3 and 82.4).

## Discussion

As discussed (13), signals from AND and EST seem to be bimodal, and primarily mediated either by the hypothalamus or by the olfactory regions. Consistent with the fact that both compounds were odorous, the conjunctive analysis showed involvement of olfactory areas even when the hypothalamic pathway predominated (table 82.2).



**Figure 82.5**  
Respiratory movements. The vertical axis shows percentage change in mean amplitude and frequency in relation to the mean baseline value. All of the groups showed an increase in amplitude and a decrease in frequency during scans compared with the respective pre-scan baselines, independently of stimulus type. Data are expressed as mean and SEM. No significant differences were observed between the groups irrespective of the stimulus type. As previously reported, the variation within each group was relatively high.

The major finding in the present study was that the preferred pathway in relation to the presented compound was associated with the responder's sexual orientation (at least in men) rather than the biological sex. This finding was based on an objective and user-independent state-of-the-art method, consistent across several types of analysis. According to the method applied, the material was sufficient to generate inference at group level, implying that each subject was representative of his or her designated group (25, 26).

The odors presented in this study have been used in several of our previous experiments (13, 19, 21, 22). To avoid the possibility that the results would rely on one specific odor, four different smells were used during the OO scans. In contrast, AND and EST were presented four times during the same scan. It might be claimed that the OO condition could produce greater activity in odor-processing areas than the pheromone conditions just because novel smells were presented during the OO-scans. However, a previous activation study with vanillin, presented in the same manner as AND and EST, showed no significant difference in the pattern or degree of activation compared with OO (19). Furthermore, the presently observed distribution and order of magnitude of the activation of olfactory regions by EST in HoM and HeW, and by AND in HeM were not consistently different from those resulting from OO. It is thus conceivable that the on-off mode of stimulus presentation prevented habituation, at least to a certain degree, thereby minimizing a potential bias due to presentation of one versus several compounds during the respective scans.

Another issue requiring clarification is that no significant clusters were found in the olfactory brain when

**Table 82.3**  
Hormone levels

Group	DHEAS, $\mu\text{mol/Liter}$	S-Testosterone, Free, nmol/Liter	S-Testosterone, nmol/Liter	S-Prolactin, $\mu\text{g/Liter}$	S-FSH, Units/Liter	S-LH, Units/Liter	S-Androstendione, nmol/Liter
HoM	$9.8 \pm 4.3$	$11.6 \pm 5.8$	$17.2 \pm 6.8$	$4.4 \pm 1.4$	$4.8 \pm 4.7$	$3.8 \pm 2.3$	$5.9 \pm 1.1$
HeM	$9.8 \pm 2.6$	$11.7 \pm 3.6$	$21.7 \pm 7.0$	$3.8 \pm 1.6$	$3.8 \pm 5.5$	$3.5 \pm 1.5$	$5.9 \pm 0.7$

DHEAS, dehydroepiandrosterone sulfate; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

**Table 82.4**  
Olfactory thresholds

Group	Butanol, M	AND, M	EST, M
HoM	$2.4 \times 10^{-5} \pm 4.0 \times 10^{-5}$	$1.5 \times 10^{-4} \pm 1.0 \times 10^{-4}$	$3.5 \times 10^{-4} \pm 2 \times 10^{-4}$
HeM	$5.0 \times 10^{-5} \pm 5.0 \times 10^{-5}$	$1.0 \times 10^{-4} \pm 0.5 \times 10^{-4}$	$1.0 \times 10^{-4} \pm 2 \times 10^{-4}$
HeW	$5.0 \times 10^{-5} \pm 1.0 \times 10^{-5}$	$1.0 \times 10^{-4} \pm 1.5 \times 10^{-4}$	$2.0 \times 10^{-4} \pm 2 \times 10^{-4}$

HoM and HeW smelled AND, or when HeM smelled EST, although both compounds were clearly perceived as odorous. That these circuits were indeed involved is, however, indicated by the emergence of clusters in the olfactory regions also in conditions showing the hypothalamic activation in the between-group conjunctural analysis (table 82.2). To address this issue more specifically, we conducted a post hoc test analyzing which activations were common for AND and EST (in relation to AIR) within each separate group of subjects. Conjunctural clusters for AND and EST were found in the amygdala, piriform cortex, and a minor portion of the anterior insula in all of the groups (Talairach's coordinates for local maxima were  $-22, +4, -14$  and  $+4, -16, +4$  in HeM;  $+26, -2, -8$  and  $+20, -14, -14$  in HeW; and  $-20, -4, -16$  in HoM).

Together, these data support the previously proposed hypothesis that odorous pheromones may act bimodally, and use two different pathways (see ref. 13 and the introduction). Furthermore, they imply that activations induced by AND and EST are partially overlapping. This overlap could explain the lack of significant clusters when contrasting the two steroids with each other and using the whole brain as search space. It applies especially to HoM, whose amygdala plus piriform cluster generated by EST also covered a minor portion of the anterior hypothalamus.

The maximal activations with AND and EST were clearly separable, reproducible (ref. 13, published data), and different from activations caused by common odors (13, 19–21). They were assessed with statistical parametric mapping statistics, which is conservative with respect to type 1 error. Furthermore, the experimental conditions were standardized and identical in all subjects. When adding to that the improbability of chance activation by AND in HoM in the brain area very similar to that in HeW, it seems convincing that we detected an undistorted physiolog-

ical response. Given the small size of the individual hypothalamic nuclei, however, it is important to emphasize that the finding of a local maximum with atlas coordinates corresponding to the location of a specific hypothalamic nucleus does not imply that only this nucleus was activated. Rather, it indicates that an area of 10 mm around this coordinate was maximally involved. At present, therefore, we can only conclude that HoM differed from HeM and resembled HeW in that their hypothalamus was activated by AND, and with the maximum in the preoptic area.

The preoptic area participates in the integration of hormonal and sensory cues that are necessary for sexual behavior. It harbors cells releasing luteinizing hormone-releasing hormone (29). In humans, these cells develop from the migrating neuroblasts of olfactory mucosa (30) and mediate estrogen feedback (31). According to a study by Dorner et al. (31), HoM respond to oestrogen injections with increased serum concentrations of luteinizing hormone (positive estrogen feedback), thus like HeW and not HeM (31). The preoptic area also harbors neuronal conglomerates (interstitial hypothalamic nuclei) whose possible sexual dimorphism in humans has been discussed (32–34). Their size in humans ( $<1 \text{ mm}^3$ ) precludes, however, further argumentation about their relevance for the present results.

The difference between HoM and HeM could reflect a variant differentiation of the anterior hypothalamus in HoM, leading to an altered response pattern. Alternatively, it could reflect an acquired sensitization to AND stimuli in the hypothalamus or its centrifugal networks, due to repeated sexual exposure to men (35). A third possibility is that HeW and HoM associated AND with sex, whereas HeM made a similar association with EST. These tentative mechanisms are not mutually exclusive, nor can they be discriminated on the basis of the present PET data.

Whether the concentrations of AND and EST used during the present experiments are relevant for physiological conditions is at present uncertain. It has been reported, however, that the neuronal response to pheromones becomes saturated already at  $10^{-8}$  M, and that the tuning curve does not broaden with increasing concentrations (36). Thus, the response may be similar in high and normal environmental concentrations. Finally, it is important to emphasize that the present study was not designed to address the issue of olfactory pathways. This said, when considering the short time course of the rCBF increase and the longer time course with humoral distribution of AND during experiments with boars (37), a chemical-sensing pathway seems much more probable than absorption into the blood stream. As to the discussion concerning the locus for nasal detection, it is of interest to note that some recent preliminary observations suggest a possibility of pheromone signal transduction through the olfactory mucosa (38, 39). A further clarification of this matter needs much more extensive investigation. Nevertheless, the differentiated pattern of cerebral activation with AND and EST compared with OO observed in the present study offers argument for the singularity of these two compounds, and strengthens the notion that signal responses from putative pheromones could operate in humans also. In addition, the colocalization of hypothalamic responses with brain circuits that are involved in human reproduction and that in animals are designed to recognize sex further indicates hypothalamic involvement in physiological processes related to sexual orientation in humans.

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## Introduction

Psychosexuality was at the cornerstone of Freud's (1905/1953) first effort to articulate a theory of child development and mental functioning. At the turn of this century, other European scholars also had much to say about childhood sexuality (e.g., Moll, 1919; cf. Kern, 1973/1974; Sulloway, 1979; Whitam, 1980). Although psychoanalytic thinking about psychosexual development continues to play an important role in contemporary theorizing and clinical work (e.g., Chodorow, 1989; Fast, 1984; Friedman, 1988; Lewes, 1988; Meyer, 1982; Person & Ovesey, 1983; Roiphe & Galenson, 1981; Scharff, 1990; Socarides, 1988; Stoller, 1968a, 1975, 1985a), the field is now much more diverse and differentiated. Over the past few decades, the study of psychosexual development has become part of the much broader framework of sex and gender research, which draws to it scholars from disciplines that cut through the social and biological sciences. Like other aspects of child psychiatry and psychology, an informed interdisciplinary approach is required to best study psychosexual development and its disorders.

This article will review current knowledge about psychosexual disorders in children and adolescents. Its orientation will be to focus on clinical-research issues that are of central concern to child psychiatrists, psychologists, and members of the allied disciplines. Within the much broader framework of sex and gender research, the scope is intentionally selective. Particular attention is given to the contributions that normative studies of gender development have for the understanding of the atypical conditions that will be discussed here (cf. Cicchetti, 1984, 1989).

## Terminology and Phenomenology

Three terms—*gender identity*, *gender role*, and *sexual orientation*—are parameters of particular use in thinking about psychosexuality (Green, 1974).

*Gender identity* can be defined in several ways. At an affective or evaluative level, gender identity refers to the child's basic sense of self as a male or a female. Al-

though not well-studied, an emotional resonance often accompanies this awareness (see, e.g., Stoller, 1964a, b, 1965, 1968b). If, for example, one "challenges" a young child regarding his or her gender identity, as is done on some of the widely used tests of "gender constancy" (e.g., Slaby & Frey 1975), it is not unusual that the response is affectively charged: "Of course I'm not a boy—I'm a girl. What a silly question!" At a cognitive level, gender identity in its most basic form, refers to the child's recognition of being a boy or a girl. Such recognition appears to be preceded by the cognitive capacity to correctly classify social markers, such as hair-length and clothing style, associated with maleness and femaleness (e.g., Etaugh, Grinnell & Etaugh, 1989; Intons-Peterson, 1988; Katcher, 1955; Leinbach & Fagot, 1986; Levin, Balistrer & Schukit, 1972; McConaghy, 1979; Thompson, 1975).

In developmental research, *gender role* refers to the child's adoption of behaviors culturally defined as masculine or feminine. In young children, this includes sex-typed toy, dress-up and role play. Preference for same versus opposite-sex peers as playmates and involvement in rough-and-tumble play and aggression are also behaviours that can be subsumed under the concept of gender role. In later childhood and adolescence, gender role can also be assessed in relation to personality dimensions (e.g., expressiveness vs instrumentality) that have stereotypic masculine or feminine connotations (see Huston, 1983, 1985).

Lastly, *sexual orientation* refers to one's preferential erotic arousal pattern. In most individuals, sexual arousal is preferentially directed either to members of the opposite sex or to members of one's own sex (heterosexual or homosexual). Most adult individuals display an erotic preference for physically mature sexual partners, but a marked disparity in age-of-partner or reliance on fetishistic stimuli are common clinical problems (e.g., pedophilia and transvestism, respectively).

Youngsters who meet the DSM-III-R (APA, 1987) criteria for the Gender Identity Disorder of Childhood display markedly deviant behaviours pertaining to gender identity and gender role. Two case vignettes illustrate the typical behavioural picture:

William, a boy approaching 4 years old with an IQ of 111, was referred because of parental and teacher concern regarding his gender identity development. Prior to the age of 2, he developed an "obsessional" interest in women's shoes and clothing. He loved to walk around in his mother's shoes. He often placed items of clothing, such as sweatpants, on his head, to simulate long hair. He often would wear his mother's T-shirts during the day and to bed. Because they were so big on him, he appeared to experience the T-shirt as a dress. At the time of the assessment, William preferred to play with girls, although his parents were concerned that at times he was so absorbed in fantasy play that he took little interest in other children. He was preoccupied with withes. He cross-dressed daily at nursery school. His toy and role interests were predominantly feminine. He only enjoyed books whose central characters were female. He showed little interest in rough-and-tumble play. Most concerning to his parents was William's repetitive insistence for the past six months that he was a girl and that he had a "hole," not a penis. During the clinical interview, William displayed a rigid, constricted preoccupation with femininity, talking about wicked witches and his belief that he was a girl.

Alice, a 4-year-old girl with an IQ of 125, was referred because of parental and teacher concern regarding her gender identity development. Around the age of 2, Alice's parents reported that she reacted with great upset to her exclusion from rough-and-tumble play by her older male cousins. Ostensibly, this was because Alice was prone to severe asthma attacks and they did not want her to get very overstimulated. In any case, Alice began to insist on wearing stereotypically masculine clothing (e.g., corduroy pants, shirts) and rejected with vehemence any clothing that was typical for girls (e.g., dresses, bows, etc.). Her toy and role interests were masculine. Her kindergarten teacher mistook her for a boy because of her physical appearance (hair style and clothing cues). What most concerned Alice's parents was her insistence that she was a boy and that she was preoccupied with acquiring a penis. She would cry when told that she could not use scissors to cut off her younger brother's penis and attach it to herself.

More detailed clinical vignettes of children with gender identity disorder may be found elsewhere (e.g., Green, 1974, 1987; Stoller, 1968c; Zuger, 1966).

The central phenomenology of these children, then, is the marked identification with the opposite sex. In both examples, the children also appeared to misclassify their sex. There is some evidence that the misclassification is age-related (Zucker, Kuksis & Bradley, 1988), but it probably also reflects the degree of gender dysphoria or discontent. In general, however, older

youngsters with gender identity problems do not misclassify their sex, but will voice the desire to be of the opposite sex or find little that is positive about their own sex.

### Developmental Patterns

Is there a developmental sequence to these three parameters of psychosexuality? Several writers have suggested that gender identity emerges first, between the age of 2–3 years, followed by the consolidation of gender role preferences during the preschool and school-age years, with the appearance of sexual orientation (eroticism) occurring around the time of puberty (e.g., Green, 1974; cf. Money, 1973; Rosen & Rekers, 1980; Stoller, 1968a).

Empirical studies have confirmed that the ability to classify males and females correctly emerges between the ages of 2 and 3 years (e.g., Leinbach & Fagot, 1986). Moreover, there is evidence that this ability appears to affect at least some aspects of sex-typed behaviour. For example, Fagot, Leinbach and Hagan (1986) tested toddlers between the ages of 21 and 40 months on a gender-labelling task in which they were required to discriminate between pictures of boys and girls. (Because correct self-labelling precedes labelling of others (see, e.g. Eaton & Von Bargen, 1981), Fagot et al. (1986) assumed that toddlers who passed this task would have some awareness of their own gender status.) Over a 4-week period, these toddlers were then observed in a naturalistic play setting in which the occurrence of a number of sex-typed behaviours was assessed. It was found that the toddlers (M age, 30 months) who "passed" the gender-labelling task spent more time playing with same-sex peers than did the toddlers (M age, 26 months) who "failed" the task; the girls who passed the task were also less aggressive than were the girls who failed it. Both behavioural differences remained significant when age was covaried. In the classical cognitive-developmental perspective of gender development (Kohlberg, 1966), correct self-labelling is viewed as an important motivational force in influencing subsequent sex role behaviour—"I am a girl, therefore I want to do girl things."

Critics of cognitive-developmental theory have argued that some aspects of gender role behaviour, particularly sex-typed toy preferences, predate the child's conscious awareness of gender identity or knowledge of sex stereotypes (e.g., Blakemore, LaRue & Olejnik, 1979; cf. Eisenberg, 1983; Eisenberg, Murray & Hite, 1982). Thus, a revisionist inversion could be made of the gender identity–gender role pathway—"It seems like I like to do girl's things, therefore I must be a girl." In any case, such data suggest that factors other than gender identity also influence the emergence of some aspects of sex-dimorphic behaviour, a perspec-

tive that is not incompatible with an integrative, multiple influence model of gender development (cf. Meyer-Bahlburg, 1984a).

In point of fact, gender identity and gender role are probably closely intertwined, particularly in early childhood. Cognitive-developmental studies of gender constancy have illuminated limitations in children's thinking about gender (e.g., the notion that sex-dimorphic activities and clothing style define one's gender identity) (see, e.g., Eaton & Von Bargen, 1981; Emmerich, Goldman, Kirsh & Sharabany, 1977; Marcus & Overton, 1978; Slaby & Frey, 1975). Thus, these two parameters have the potential for reciprocal influence. Perhaps the main lesson to be drawn from studies in the cognitive-developmental tradition is that the child's apparent predilection to organize information along a gender axis (see Maccoby, 1988) strongly influences the child's processing and understanding of social phenomena (see, e.g., Martin & Halverson, 1981, 1987; Stangor & Ruble, 1987).

Although students of normative gender development have made some contributions to the study of child and adolescent sexuality (see Serbin & Sprafkin, 1987), little attention has been given to the determinants of sexual orientation other than to note, perhaps, the temporal association between gender identity and sexual orientation and the implicit acceptance that conventional gender identity development is associated with later heterosexuality (e.g., Fagot, 1989). The bulk of research on sexual orientation development comes from quarters outside the domain of academic child development research (see section on Etiological Models).

## Epidemiology

### Prevalence and Incidence

There are no formal prevalence studies of children with gender identity disorder. There are also no empirical data regarding changes in the incidence of gender identity disorder over the past several decades. Meyer-Bahlburg (1985) has characterized gender identity disorder as a "rare phenomenon." There is little doubt that gender identity disorder is an uncommon child psychiatric condition, like, say, autism, and could in no way compete in a prevalence race with conditions such as attention deficit disorder or conduct disorder.

If one relies on prevalence estimates from the "parent" condition, transsexualism, then one summary account suggests an occurrence of 1 in 24,000–37,000 men and 1 in 103,000–150,000 women (see Meyer-Bahlburg, 1985). This method suffers, however, from at least two problems: first, it is based on the number of persons attending clinics that serve as gateways for

surgical and hormonal sex reassignment, which may not see all gender-dysphoric adults. Second, the assumption that children with gender identity disorder will, in fact, be later diagnosed as transsexual is not necessarily true. Nevertheless, prevalence estimates of gender identity disorder derived from data on adult transsexualism support the notion of its rarity.

As will be noted in the section on follow-up, gender identity disorder in childhood is strongly associated with subsequent homosexuality. Accordingly, the prevalence of gender identity disorder might also be derived from the literature on the epidemiology of homosexuality. The prevalence of homosexuality, however, remains a hotly contentious issue, often inextricably bound up with "sexual politics" (see, e.g., D'Emilio, 1984). It is fairly well-recognized that homosexual experiences are not equivalent to preferential homosexuality. Thus, the oft-cited finding by Kinsey, Pomeroy and Martin (1948, p. 623) that 37% of adult males in the U.S.A. had had at least one postpubertal homosexual experience leading to orgasm is not of great epidemiological relevance. Even when one attempts to use the Kinsey data set or its variants to estimate the prevalence of preferential homosexuality, there is considerable disagreement. Voeller (1990) recently concluded that "an average of 10% of the population (men and women combined) could be designated as Gay (homosexual)" (*italics omitted*). Other scholars who have reworked the Kinsey terrain suggest lower prevalence rates, typically between 2 and 6% (e.g., Fay, Turner, Klassen & Gagnon, 1989; Gebhard, 1972; Whitam & Mathy, 1986).

Using prevalence data on homosexuality to gauge the prevalence of childhood gender identity disorder is based on retrospective studies that have shown a substantial proportion of homosexual men and women to recall greater rates of childhood cross-gender behaviour than their heterosexual counterparts (e.g. Bell, Weinberg & Hammersmith, 1981; Harry, 1982; Saghir & Robins, 1973; Whitam & Mathy, 1986). Bell et al.'s (1981) large-scale study of heterosexual and homosexual men and women in the San Francisco Bay Area found that the developmental variable labelled "gender nonconformity" (a term that seems to refer primarily to behaviours pertaining to gender identity and, particularly, gender role) was, among men, "first in importance among...15 developmental variables" (p. 76) in predicting subsequent sexual orientation. Among women, it was second in importance. Nevertheless, these studies pose their own interpretive problems. For example, the specific behaviours assessed vary from one study to the next. How one should determine whether or not an individual was cross-gendered vs not cross-gendered ("caseness") is rarely specified; moreover, individuals classified as cross-gendered would not

necessarily meet the complete diagnostic criteria for the gender identity disorder of childhood (APA, 1987).

More liberal estimates of prevalence can be judged from studies of children in whom specific cross-gender behaviours have been assessed (see, e.g., Zucker, 1985, pp. 87–95). For example, on the Child Behaviour Checklist (CBCL) (Achenbach & Edelbrock, 1981), a factor-analytically derived parent-report behaviour problem questionnaire, two items pertain to cross-gender identification: “behaves like opposite sex” and “wishes to be of opposite sex.” In the standardization study, endorsement of both items was more common for girls than for boys, regardless of age and clinical status (referred vs non-referred). Among referred boys, the desire to be of the opposite sex was quite high for the 4–5-year-olds—15.5%—but dropped off sharply thereafter. Among referred girls, the desire to be of the opposite sex seemed more stable (range, 4.2–8.3%) and was consistently higher than that of the non-referred girls.

The main problem with such data is that they do not adequately identify patterns of cross-gender behaviour that would be of use in determining “caseness.” Thus, such methods of data collection may be best viewed as screening devices for more intensive evaluation (cf. Pleak, Meyer-Bahlburg, O’Brien, Bowen & Morganstein, 1989).

### Referral Rates

Consistently, it is observed that boys are referred more often than are girls for concerns regarding gender identity. Since its inception in 1978, the first author’s clinic in Toronto, Canada has had a referral ratio of 5.25:1 ( $N = 175$ ) of boys to girls.

How might this disparity be best understood? It may be that the true prevalence of psychosexual disorders is greater in males, perhaps due to a greater biological vulnerability. For example, it has been noted that, among mammals, development along male lines is dependent on the production of androgen during early fetal development. If appropriate androgen secretion does not occur, or if cell receptors do not respond to circulating androgen, then fetal development proceeds along female lines. The androgen-insensitivity (testicular feminization) syndrome (see, e.g., Perez-Palacios, Chavez, Mendez, Imperato-McGinley & Ulloa-Aguirre, 1987) in genetic males is the most poignant illustration of this postulate. Accordingly, it has been suggested that male fetal development is more “complex” and thus more susceptible to errors that may affect postnatal psychosexual genesis (e.g., Eme, 1979; Gadpaille, 1972; Money & Ehrhardt, 1972; Stoller, 1972).

Regardless of the contribution of biological events, social factors also appear to play a role in accounting

for the disparity in referral rates. For example, there is less tolerance of cross-gender behaviour in boys than in girls, from both peers and adults (e.g., Fagot, 1977, 1985; Green, Williams & Harper, 1980; Zucker, Wilson & Stern, 1985). Adults are also more likely to predict atypical outcomes, such as homosexuality, in feminine boys than in masculine girls (Antill, 1987; Martin, 1990). Thus, depending on one’s point of view, it could be argued that boys are overreferred or that girls are underreferred. Clinically, there is evidence that girls may be required to display more extreme cross-gender behaviour before parents seek out a clinical assessment. For example, Zucker (1989) reported that mothers of gender-referred girls ( $N = 19$ ) were significantly more likely to rate the two CBCL items pertaining to gender identity as a “2” (on a 0–2 point scale) than were the mothers of gender-referred boys ( $N = 108$ ), 57.8% versus 25.0%, respectively.

### Diagnosis

The diagnosis of Gender Identity Disorder of Childhood appeared in the psychiatric nomenclature for the first time in the DSM-III (APA, 1980). The diagnosis derived largely from the work of Green (1974). There were revisions to the criteria, particularly for girls, in the DSM-III-R (APA, 1987), and the DSM-IV Subcommittee on Gender Identity Disorders and Transsexualism has recommended further revisions (Bradley et al., 1991). [The ICD-10 diagnoses pertaining to gender identity disorders have utilized much of the descriptive terminology in DSM-III and III-R (see Rutter, 1989).] This section will consider some of the central diagnostic issues pertaining to the DSM-III-R criteria for gender identity disorder, which are shown in table 83.1 (for a more complete consideration of diagnostic issues, see Zucker, 1982, 1985, 1990b, 1992).

For both sexes, it can be seen that two criteria (Points A and B) are required for the diagnosis. Point A seems to index the child’s sense of discomfort with assigned sex and the desire to change sex. Descriptively, this criterion is most directly tied to the concept of gender identity. Point B-1 emphasizes the child’s preference for cross-gender roles and activities and, at the same time, the child’s aversion to roles and clothing more typically associated with one’s own sex. Point B-2 emphasizes the child’s dislike and aversion of one’s sexual anatomy.

### Sex Similarities and Differences in the DSM-III-R Criteria

For both sexes the child must show a “persistent and intense distress” about being a boy or a girl (see table 83.1). This phrase did not appear in the DSM-III criteria for either sex; moreover, DSM-III-R does not spec-

**Table 83.1**

DSM-III-R diagnostic criteria for gender identity disorder of childhood

For females:

- (A) Persistent and intense distress about being a girl, and a stated desire to be a boy (not merely a desire for any perceived cultural advantages from being a boy), or insistence that she is a boy.
- (B) Either (1) or (2):
  - (1) persistent marked aversion to normative feminine clothing and insistence on wearing stereotypical masculine clothing, e.g. boys' underwear and other accessories
  - (2) persistent repudiation of female anatomic structures, as evidenced by at least one of the following:
    - (a) an assertion that she has, or will grow, a penis
    - (b) rejection of urinating in a sitting position
    - (c) assertion that she does not want to grow breasts or menstruate
- (C) The girl has not reached puberty.

For males:

- (A) Persistent and intense distress about being a boy and an intense desire to be a girl or, more rarely, insistence that he is a girl.
- (B) Either (1) or (2):
  - (1) preoccupation with female stereotypical activities, as shown by a preference for either cross-dressing or simulating female attire, or by an intense desire to participate in the games and pastimes of girls and rejection of male stereotypical toys, games, and activities
  - (2) persistent repudiation of male anatomic structures, as indicated by at least one of the following repeated assertions:
    - (a) that he will grow up to become a woman (not merely in role)
    - (b) that his penis or testes are disgusting or will disappear
    - (c) that it would be better not to have a penis or testes
- (C) The boy has not yet reached puberty.

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ify how one assesses such distress or in what ways it might be distinct from other operationalized components in the criteria. For example, how does it differ from the "desire" to be of the opposite sex, which constitutes the remaining portion of the Point A criterion?

During the development of DSM-III, the concept of "distress" played a formative role in describing the nature of mental disorder (Spitzer & Endicott, 1978), and this probably influenced the inclusion of the passage "persistent and intense distress." It is unclear, however, to what extent young children with marked cross-gender identification experience internalized distress over their "wish" to be of the opposite sex. For example, a 3-year-old boy who insists that he is a girl and cross-dresses on a daily basis may not experience felt distress until the behaviour is interfered with (cf. Stoller & Newman, 1971). In any case, application of the distress criterion to children may be insensitive to developmental issues—there are probably many child psychiatric disorders in DSM-III-R in which the child does not experience felt distress, or if it occurs, is context-dependent. Given the complexities of the distress concept, the DSM-IV Subcommittee on Gender Identity Disorders and Transsexualism (hereafter, the DSM-IV Subcommittee) has recommended that the passage pertaining to "persistent and intense distress" be deleted (Bradley et al., 1991).

As noted in detail elsewhere (Zucker, 1992), the phraseology that follows the distress portion of the Point A criterion shows a subtle difference between the sexes. For girls, there must be a "*stated* desire to be a boy" (emphasis added), whereas for boys, there must be an "*intense* desire to be a girl" (emphasis added). As

well, the passage for girls contains no reference to intensity or some other descriptor pertaining to chronicity. Zucker (1992) noted that the basis for this distinction was unclear and was particularly confusing since the phraseology had been identical for the two sexes in the DSM-III: "strongly and persistently *stated* desire to be a boy (girl)" (emphasis added). For boys, how one infers such desire independent of other aspects of the criteria, such as the "preoccupation with female stereotypical activities" in Point B-1, is critical if specific reliance on verbal statements is not required. The DSM-IV Subcommittee has recommended that this particular criterion be identical for boys and girls and be based on verbal statements of a persistent nature (Bradley et al., 1991).

It can also be seen in table 83.1 that the Point A criterion for girls contains an additional proviso—that the desire to be a boy is not "merely a desire for any perceived cultural advantages from being a boy." Although the motivational basis for a girl's desire to be a boy may have relevance for such parameters as natural history and response to treatment, it is not clear why it should be used diagnostically. It could be argued that boys who wish to be girls perceive similar, albeit inverted, cultural advantages (e.g., "girls can wear dresses *and* pants", "girls don't have to fight, boys do") (cf. Green, 1974). Accordingly, the DSM-IV Subcommittee has recommended that this exclusion criterion be deleted (Bradley et al., 1991).

The Point B-1 criterion included some of the cross-gender behaviours commonly observed in boys with gender identity disorder, such as cross-dressing and cross-gender toy play. In addition, this criterion included an aversion to culturally typical masculine

**Table 83.2**  
DSM-IV draft criteria for gender identity disorder

- 
- (A) A profound and persistent cross-gender identification.  
In children, as manifested by at least 4 of the following:
- (1) repeatedly stated desire to be, or insistence that he or she is, the opposite sex
  - (2) in girls, insistence on wearing stereotypical masculine clothing; in boys, preference for cross-dressing or simulating female attire
  - (3) strong and persistent preferences for cross-sex roles in fantasy play or persistent fantasies of being the opposite sex
  - (4) intense desire to participate in the games and pastimes of the opposite sex
  - (5) strong preference for playmates of the opposite sex
- In adolescents and adults, as manifested by symptoms such as a stated desire to be the opposite sex, frequent passing as the opposite sex, desire to live as or be treated as the opposite sex, or the conviction that one has the typical feelings and reactions of the opposite sex
- (B) Persistent discomfort with one's assigned sex or sense of inappropriateness in that gender role.  
In children, manifested by any of the following:
- In boys, assertion that his penis or testes are disgusting or will disappear, or assertion that it would be better not to have a penis, or aversion towards rough-and-tumble play and rejection of male stereotypical toys, games, and activities
- In girls, rejection of urinating in a sitting position or assertion that she does not want to grow breasts or menstruate, assertion that she has or will grow a penis, or persistent marked aversion towards normative feminine clothing
- In adolescents and adults, manifested by symptoms such as preoccupation with getting rid of one's primary and secondary sex characteristics (e.g. request for hormones, surgery, or other procedures to physically alter sexual characteristics to simulate the opposing sex) or belief that one was born the wrong sex.
- For sexually mature individuals, specify history of sexual attraction: towards males, females, both, neither, unspecified
- 

*Note.* From Bradley et al. (1991).

activities. Thus, there was an emphasis on a cross-gender preference, not an equal attraction for both masculine and feminine activities, which would be somewhat more difficult to interpret. Interestingly, the examples provided in DSM-III-R did not include a preference for female roles in fantasy play or a preference for female playmates, which are also extremely common traits among cross-gender identified boys (Green, 1976).

In the DSM-II (APA, 1980), a similar criterion was not included for girls, i.e. it made no reference to the gender-disturbed girl's intense preoccupation with cross-dressing and involvement in masculine stereotypical activities. In DSM-III-R, however, a new criterion was included (B-1), which emphasized the gender-disturbed girl's aversion to normative feminine clothing and involvement in cross-dressing. The inclusion of this criterion was based, in part, on clinical observation that an aversion to normative feminine clothing may distinguish gender-disturbed girls from girls who engage in masculine behaviour but are not necessarily gender dysphoric (Zucker, 1982).

The Point B-2 criterion for boys captured the concept of "anatomic dysphoria," that is, the dislike of one's sexual anatomy. The behavioural manifestations remained unchanged from those given in DSM-III. The wording of the criteria for girls, however, changed substantially from that of DSM-III. The DSM-III criteria required either "delusional" (e.g., that "she has... a penis") or "immature" thinking but did not include feelings of dislike toward one's sexual anatomy (Zucker, 1982). This changed in DSM-III-R and the criteria for girls became similar to the criteria for boys.

In general, the DSM-IV Subcommittee has supported the idea that the phenomenology of gender

identity disorder is more similar than different between boys and girls. Accordingly, it has recommended that more attention be paid to this in the criteria (Bradley et al., 1991).

#### **Distinctness of the A and B Criteria**

The theoretical basis for requiring two distinct criteria was never formally articulated in DSM-III (APA, 1980), although it seemed related, in part, to separating the concepts of gender identity and gender role. Empirical evidence, however, suggests that these two facets of gender identification are strongly intercorrelated (see, e.g. Bentler, Rekers & Rosen, 1979; cf. Rosen, Rekers & Friars, 1977).

Research utilizing the DSM-III and DSM-III-R criteria found that younger children (mean age, 6.4 years,  $N = 54$ ) were significantly more likely to receive the diagnosis than were older children (mean age, 9.0 years,  $N = 54$ ) (Zucker, 1992; Zucker, Finegan, Doering & Bradley, 1984). This seemed mainly due to older children not meeting the Point A criterion. Although there was marked clinical evidence of cross-gender identification, these children did not frequently voice the desire to be of the opposite sex. In part, this seemed a function of social desirability and fear of stigma (cf. Bentler, 1976).

The DSM-IV Subcommittee considered this issue and recommended that the verbal desire to be of the opposite sex no longer be listed as a distinct criterion, but rather be one of a series of behavioural criteria that is required to meet the threshold for the diagnosis, i.e. it will no longer be a necessary condition for the diagnosis (Bradley et al., 1991). Table 83.2 shows the working draft criteria for the DSM-IV diagnosis of Gender Identity Disorder.

## Differential Diagnosis

**Children** Several differential diagnostic issues need to be considered among children. There is a type of cross-dressing in boys that appears to be qualitatively different from the type of cross-dressing that characterizes gender identity disorder. In the latter, cross-dressing typically involves outerwear (e.g., dresses, shoes and jewellery) that helps enhance the fantasy of being like the opposite sex. In the former, cross-dressing involves the use of undergarments, such as panties and nylons (Scharfman, 1976; Stoller, 1985b). Clinical data show that such cross-dressing is not accompanied by other signs of cross-gender identification; in fact, the appearance and behaviour of boys who engage in it are conventionally masculine. Clinical experience also suggests that this type of cross-dressing has some sort of self-soothing function. Many male adolescents and adults who display Transvestic Fetishism (APA, 1987) recall this form of cross-dressing during childhood (Bradley & Zucker, 1984, 1990). It should be noted, however, that prospective study has not verified the assumption that this behaviour pattern is fully contiguous with later transvestism.

When all the clinical signs of gender identity disorder are present, it is not difficult to make the diagnosis (cf. Zucker et al., 1984). But the clinician who accepts the notion that there is a spectrum of cross-gender identification must be prepared to identify what Meyer-Bahlburg (1985) described as the "zone of transition between clinically significant cross-gender behavior and mere statistical deviations from the gender norm" (p. 682). Clinical experience suggests that boys who fall into this ambiguous zone do poorly in male peer groups, avoid rough-and-tumble play, are disinclined toward athletics and other conventionally masculine activities, and feel somewhat uncomfortable about being male; however, these boys do not wish to be girls and do not show an intense preoccupation with femininity. Friedman (1988) coined the term "juvenile unmasculinity" to describe such boys, who, he argued, suffer from a "persistent, profound feeling of masculine inadequacy which leads to negative valuing of the self" (p. 199). It is not clear whether this behaviour pattern actually constitutes a distinct syndrome or is simply a mild form of gender identity disorder; in any case, the residual diagnosis Gender Identity Disorder Not Otherwise Specified (APA, 1987) could be employed in such cases.

In girls, the primary differential diagnostic issue concerns the distinction between gender identity disorder and "tomboyism." The study of a community sample of tomboys by Green, Williams and Goodman (1982) showed that these girls shared a number of the

cross-gender traits observed in clinic-referred gender-disturbed girls (Zucker, 1982, 1989). In part, the DSM-III-R criteria for gender identity disorder in girls were modified in the hope of better differentiating these two groups of girls. At least three characteristics may be most useful in making the differential diagnosis: (1) by definition, girls with gender identity disorder indicate an intense unhappiness with their status as females, whereas this should not be the case for tomboys; (2) girls with gender identity disorder display an intense aversion to the wearing of culturally defined feminine clothing under any circumstances, whereas tomboys do not manifest this reaction with the same intensity, though they may prefer to wear casual clothing, such as jeans; (3) girls with gender identity disorder, unlike tomboys, manifest a verbalized or acted-out discomfort with sexual anatomy.

The final differential diagnostic issue concerns children and adolescents with physical intersex (hermaphroditic) conditions. In the DSM-III (APA, 1980), an intersex condition was an exclusion criterion for the adult diagnosis of Transsexualism; however, it was not an exclusion criterion for the Gender Identity Disorder of Childhood, although it was noted that "physical abnormalities of the sex organs are rarely associated with gender identity disorder" (APA, 1980, p. 256). In the DSM-III-R (APA, 1987), this exclusion criterion was eliminated for Transsexualism.

As noted by Meyer-Bahlburg (1991), neither DSM-III or DSM-III-R contained substantive information regarding the conceptual issues. The DSM-III-R has not explicitly addressed the question of whether children who display significant cross-gender identification and have an intersex condition should be given a diagnosis such as gender identity disorder.

Meyer-Bahlburg (1991) has pointed out that the intersex case report literature has rarely attempted to utilize formally DSM criteria in describing patients with gender identity and gender role conflicts. Thus, among intersex patients with gender identity problems, it is not possible to specify what percentage actually would meet formal criteria for gender identity disorder or transsexualism. Meyer-Bahlburg (1991) also has argued that it is unclear if the phenomenology surrounding gender identity concerns is the same as it is observed in non-intersex persons with gender identity problems (see also Bradley et al., 1991).

Even if the phenomenology in intersex and non-intersex youngsters is similar, there do appear to be substantial differences in other variables, including prevalence, sex ratio, and associated features (Meyer-Bahlburg, 1991). It is likely, therefore, that the etiology for gender identity disorder is different for the two groups and whether treatment would be similar is an

open question. Thus, there may be a risk in applying the same diagnosis as that used with physically normal children. On the other hand, the risk is reduced if it is recognized that a particular diagnosis does not dictate identical treatment across cases. From an etiological standpoint, the use of Axis III for Physical Disorders or Conditions would be important in noting the role of the physical anomaly, but this would not preclude using the gender identity disorder diagnosis if clinically justified (for further discussion, see Bradley et al., 1991; Meyer-Bahlburg, 1991).

**Adolescents** During the adolescent years, there are at least four types of psychosexual diagnostic conditions to consider. Children with an unresolved gender identity disorder are at risk for Transsexualism (APA, 1987). The DSM-III-R diagnostic criteria specify a period of at least two years during which there is a "persistent preoccupation" with getting rid of one's primary and secondary sex characteristics. Diagnostically, it is important to assess the fixedness of the desire to change sex, since clinical management decisions will be influenced by the relative intractability of the condition. Although its validity as a syndrome has not been studied, the diagnosis Gender Identity Disorder of Adolescence or Adulthood, Nontranssexual Type (APA, 1987) can be used for gender dysphoric individuals who do not meet the complete diagnostic criteria for Transsexualism.

A second type of psychosexual problem occurs among adolescents who have had a history of gender identity disorder or its subclinical variants (cf. Friedman, 1988). These adolescents continue to show signs of cross-gender identification but do not acknowledge a homosexual orientation or profess the desire to change sex. They are often referred because of continued social ostracism. The degree of felt distress varies with regard to the continued cross-gender identification (Bradley & Zucker, 1984, 1990). It is unlikely that any formal DSM-III-R diagnosis would apply in such cases, although the residual diagnosis Gender Identity Disorder Not Otherwise Specified could be employed to indicate that the adolescent continues to struggle with gender identity issues.

A third type of psychosexual problem characterizes adolescents who are parent- or self-referred because of homosexual behaviour or orientation. Many of these youngsters have a history of gender identity disorder or a variation of it. The reason for referral varies, but from a differential diagnostic standpoint it is important to rule out continuing problems that centre on gender identity. For those youngsters who are distressed about their sexual orientation, the diagnosis Sexual Disorder Not Otherwise Specified (APA, 1987) can be given.

The last type of psychosexual problem concerns adolescent boys who cross-dress, in part, for the purpose of sexual arousal. The extent of the cross-dressing varies. In its full form, the diagnosis Transvestic Fetishism (APA, 1987) can be applied. These boys appear to have a nascent heterosexual orientation and appear unremarkably masculine in their demeanor. A history of gender identity disorder is not part of the clinical picture, but some of these boys think about sex-reassignment surgery and are at risk for Transsexualism. In DSM-III-R, transsexuals with this type of developmental history typically have a heterosexual orientation (Blanchard, Clemmensen & Steiner, 1987).

The DSM-IV Subcommittee has taken the position that the Gender Identity Disorder of Childhood, Transsexualism, and Gender Identity Disorder of Adolescence or Adulthood, Nontranssexual Type are not qualitatively distinct disorders, but reflect differences in both developmental and severity parameters. As a result, the DSM-IV Subcommittee has recommended one overarching diagnosis, Gender Identity Disorder, that can be used, with appropriate variations in criteria, across the life cycle (Bradley et al., 1991) (see table 83.2).

### Assessment

Demonstrating differences between groups with regard to presenting characteristics is a first step in validating a psychiatric syndrome (Rutter, 1978). For some child psychiatric conditions, such as hyperactivity, this has been no easy task (Prior & Sanson, 1986; Schachar, 1991). In contrast, demonstration of discriminant validity regarding many of the putative characteristics of children, mainly boys, with gender identity disorder has been fairly straightforward. Structured parent interviews regarding specific sex-typed behaviours (Green, 1987; Roberts, Green, Williams & Goodman, 1987), parent-report on sex-typed play and behaviour questionnaires (Bates & Bentler, 1973; Bates, Bentler & Thompson, 1973; Green, 1976, 1987; Zucker, Bradley, Corter, Doering & Finegan, 1980; Zucker, Bradley, Doering & Lozinski, 1985), measurement of overt (Doering, Zucker, Bradley & MacIntyre, 1989; Green, Fuller, Rutley & Hendler, 1972; Rekers & Yates, 1976; Zucker, Doering, Bradley & Finegan, 1982) and covert (Green & Fuller, 1973; Zucker, Doering, Bradley, Alon & Lozinski, 1984) sex-typed play in standardized situations, observation of sex-typed motoric behaviour (Bates, Bentler & Thompson, 1979; Green, Neuberg & Finch, 1983), assessment of gender constancy development (Zucker et al., 1988), and sex-typed indices on projective tests, including the Draw-a-Person (Green, Fuller & Rutley, 1972; Skilbeck, Bates & Bentler, 1975; Zucker, Finegan, Doering &

Bradley, 1983), the IT Scale for Children (Green et al., 1972), and the Rorschach (Zucker, Lozinski, Bradley & Doering, 1992) have all yielded differences between gender-referred probands and comparison groups, which have included sibling, psychiatric and normal controls (see also Rekers & Morey, 1989a, 1989b, 1990; Rekers, Rosen & Morey, 1990).

Although each of these forms of measurement are vulnerable to both false positives and false negatives, the overall patterning of behaviour has led, at times, to excellent group classification. Green (1987), for example, found that a discriminant function analysis required 6 of 16 sex-typed behaviours to classify correctly all boys as members of either the "feminine" group or the male control group.

An important assessment issue concerns the mediating role of age. Clinical observation has suggested that cross-gender behaviour shows a reduction with age (Green, 1975; Zuger, 1978), and, indeed, there is some empirical evidence of such a reduction (Bates, Skilbeck, Smith & Bentler, 1974; Zucker et al., 1984). The interpretive issue is why such a reduction occurs. Does it reflect a bona fide shift in the child's cross-gender-identification? Does it mean that the child's cross-gender behaviour is going "underground" as he or she becomes more aware of negative social responses by significant others? Is the problem a measurement issue in that the techniques used to assess gender identity and gender role in older children are either inappropriate or too transparent to elicit valid responses?

On a priori grounds, all of these explanations are plausible. Empirically, it has been shown that some measures are more strongly affected by age (e.g., overt sex-typed play in a structured clinic setting) than are others (e.g., sex-typed fantasy play and other projective measures) (see Zucker, 1992). This suggests that some measures of gender-role preference are probably more valid than are others for use with older children. On the other hand, Zucker et al. (1984) reported that even with age co-varied, older children referred for gender identity concerns displayed less cross-gender behaviour than did younger children on some measures.

### Associated Psychopathology

Several studies, almost exclusively with gender-problem boys, have attempted to assess their general psychological functioning. Diverse measurement approaches have been employed, including standardized behaviour problem questionnaires (Bates et al., 1973, 1979; Coates & Person, 1985; Rekers & Morey, 1989b,c; Zucker, 1985, 1990a), ratings of social behaviour in structured situations (Bates et al., 1979) and on standardized questionnaires (Zucker, 1985), assessment

of personality functioning and structure on projective tests (Goddard, 1986; Ipp, 1986; Kolers, 1986; Tuber & Coates, 1989), and ascertainment of other psychiatric disorders (Coates & Person, 1985; Lowry & Zucker, 1991). These studies point to the conclusion that boys referred clinically for gender identity problems also show, on average, a greater degree of general psychopathology than do sibling and normal controls, and the extent of psychopathology appears to be commensurate with that of other clinically-referred youngsters who are demographically comparable.

The most detailed analysis of general behaviour problems as discerned by parent-report on the CBCL has been described by Zucker (1990a). Boys with gender identity disorder ( $N = 77$ ) had indices of psychopathology at a level similar to that of the clinic-referred group in Achenbach and Edelbrock's (1983) standardization sample. Elsewhere, Zucker (1985) reported that children with gender identity disorder had significantly more behaviour problems on the CBCL than did their siblings, but did not differ when compared to concurrently assessed, demographically matched, clinical controls. (In these analyses, all CBCL items rated positive pertaining to gender behaviour were set to 0 so as to avoid artificial inflations of the general psychopathology indices.) Zucker (1990a) also found that boys with gender identity disorder had significantly higher Internalizing T scores than Externalizing T scores on the CBCL. The predominance of overcontrolled psychopathology is consistent with findings from other investigators using a different behaviour problem questionnaire (Rekers & Morey, 1989c; Sreenivasan, 1985), observational ratings of "body constriction" (Bates et al., 1979), and clinical diagnoses of separation anxiety and dysthymia (Coates & Person, 1985).

Given the presence of other forms of psychopathology, what connection, if any, do these difficulties have with the gender identity disorder itself? On this matter, there have been several views, which are summarized in table 83.3.

The first view holds that the child's marked cross-gender behaviour is the target of social ostracism, particularly by peers, which is the mechanism that leads to the display of general psychopathology (Rekers, 1977; cf. Green, 1974; Green et al., 1980).

The second view is somewhat more complex, implicating the role of parental influences, such as psychiatric disorder, erratic care-giving, and marital discord on the child's gender development. In general, this perspective attempts to consider the genesis of the gender identity disorder in the context of more global problems in the child's development and familial psychopathology (see, e.g. Bates et al., 1974). Coates (1985, 1990) and her colleagues (e.g. Coates, Friedman & Wolfe, 1991; Coates & Person, 1985; Marantz &

**Table 83.3**

Possible relations among gender identity disorder, general psychopathology, and parental influences in boys

- 
- (1) Gender Identity Disorder → General Psychopathology  
 (2) Parental Influences → General Psychopathology → Gender Identity Disorder  
 (3) Parental Influences<sup>a</sup> → General Psychopathology  
     Parental Influences<sup>b</sup> → Gender Identity Disorder → General Psychopathology
- 

Coates, 1991; Rainbow, 1986), who have discussed this perspective in some detail, have advanced a specific hypothesis, namely that separation anxiety—which is activated by uneven maternal availability—plays a pivotal role in the emergence of gender identity disorder in boys. According to Coates and Person (1985), severe separation anxiety precedes the feminine behaviour, which emerges in order “to restore a fantasy tie to the physically or emotionally absent mother. In imitating “Mommy” [the boy] confuse[s] ‘being Mommy’ with ‘having Mommy’. [Cross-gender behaviour] appears to allay, in part, the anxiety generated by the loss of the mother” (Coates & Person, 1985, p. 708).

The last view suggests that parental influences play a role in both general psychopathology and gender identity disorder, but that different aspects of parental functioning are involved. For example, the relation between parental influences and general psychopathology in boys with gender identity disorder could be explained by aspects of parental functioning that are, diagnostically speaking, nonspecific, such as marital discord and psychiatric disorder (e.g., Emery, 1982, 1988; Grych & Fincham, 1990). Other parental variables, such as reinforcement or tolerance of feminine behaviour and atypical psychosexual parental traits, which may be diagnostically specific (cf. Green, 1987), lead directly to gender identity disorder which, in turn, is associated with general psychopathology through the mechanism of social ostracism. Although this view suggests that gender identity disorder and general psychopathology are influenced by orthogonal mechanisms, it could also be argued that these two facets of parental influence might be related; for example, parents with extensive marital discord or personality disorder may be less mobilized psychologically to intervene in their child’s cross-gender behaviour, which increases the likelihood of subsequent exposure to social ostracism.

Unfortunately, there is only limited empirical data available on which to evaluate these different views. Because the third view has not been adequately studied empirically, it will not be discussed further. Zucker (1990a) has provided some evidence that supports the first model. It was hypothesized that extent of general psychopathology would be correlated with age, since one would expect social ostracism to exert a stronger effect over time. Using CBCL indices as measures of general psychopathology (which are standardized

for age), age was, in fact, significantly correlated with degree of CBCL psychopathology in a sample of 77 gender-disturbed boys ( $r$ s ranged from 0.27–0.37). Interestingly, age was more strongly correlated with CBCL psychopathology in the subgroup of boys ( $N = 38$ ) who met the complete DSM-III criteria for gender identity disorder (mean age, 6.4 years;  $r$ s ranged from 0.57–0.66) as compared to the subgroup of boys ( $N = 39$ ) who did not meet the complete DSM-III criteria (mean age, 9.1 years;  $r$ s ranged from 0.14–0.24). Although the extent to which the boy was called a “sissy,” as gauged by maternal report, was equally correlated with CBCL psychopathology in the two diagnostic subgroups, multiple regression analysis showed that age was the best predictor of psychopathology in the subgroup that met the complete DSM-III criteria whereas being called a “sissy” was the best predictor of psychopathology in the non-DSM-III group. Zucker (1990a) suggested that younger children probably had less familiarity with the abstract label “sissy” than did the older children, which may have accounted, in part, for its relatively stronger impact on the non-DSM-III group which was, as noted above, almost three years older than the DSM-III group.

Only limited evidence is available in support of the second model. At least two lines of evidence are required. First, it is necessary to show that there is a greater degree of parental psychopathology and family dysfunction in boys with gender identity disorder than in families of controls. Then, evidence must be given to show how such putative psychopathology influences specifically the genesis of the gender identity disorder.

On the first point, there are a few empirical studies that have provided the relevant information. Using standardized interview and self-report measures, Marantz and Coates (1991) found that mothers of boys with gender identity disorder showed more signs of psychopathology than did the mothers of demographically matched normal boys, including more pathological ratings on the Diagnostic Interview for Borderline Patients and the Beck Depression Inventory. Using the Structured Clinical Interview for DSM-III, Wolfe (1990) reported a high rate of psychiatric disorder in both the mothers and fathers of boys with gender identity disorder, although the strength of this study was limited by the absence of a control group. Other studies of boys with gender identity disorder also impli-

cate family dysfunction, as judged by rates of parental separation and divorce (Coates, 1985; Rekers & Swihart, 1989), prior parental contact with the mental health profession (Rekers, Mead, Rosen & Brigham, 1983), and parental overprotectiveness (Bates et al., 1974). Surprisingly, marital discord has not been systematically studied although ratings of relative parental dominance have not yielded between group differences (Green, 1987; Thompson, Bates & Bentler, 1977).

These data give tentative support for the hypothesis that clinic-referred boys with gender identity disorder come from families in which there is, on average, greater levels of parental and familial dysfunction than in normal controls. This conclusion, however, must be viewed with much caution. Only Marantz and Coates (1991) employed a concurrent, demographically matched control group. The absence of a clinical control group in their study leaves open the question of specificity of maternal dysfunction vis-à-vis gender identity disorder. If, for example, similar levels of maternal psychopathology were found in a clinical control group, it would seem that one could conclude, at best, that such risk factors play a non-specific role in the development of gender identity disorder (cf. Marantz, 1984).

As noted earlier, the specific role of separation anxiety disorder vis-à-vis gender identity disorder has been one psychological pathway invoked to explain the role of parental dysfunction. Coates and Person (1985) developed this argument based in part on their finding that of 25 boys with a DSM-III diagnosis of gender identity disorder, 15 (60%) also met DSM-III criteria for separation anxiety disorder.

Elsewhere, Zucker and Green (1991) noted several methodological and interpretive caveats about the Coates and Person (1985) data. First, the interview procedure used to assess separation anxiety was not described and information on interrater reliability was not given. Moreover, the diagnoses of separation anxiety and gender identity disorder were made concurrently at the time of assessment, so it is unclear whether separation anxiety actually preceded the emergence of gender identity disorder, as called for by the model. On this point, there is only clinical impression, not empirical verification. As noted by Marantz and Coates (1991), it remains unclear why cross-gender behaviour would follow the emergence of separation anxiety, since not all boys with separation anxiety disorder develop gender identity disorder and not all boys with gender identity disorder develop separation anxiety disorder.

Despite these methodological and interpretive problems, Coates and Person's (1985) diagnostic impression regarding the high rate of separation anxiety disorder is consistent with the more general finding

noted earlier that boys with gender identity disorder show a predominance of internalizing psychopathology (Zucker, 1990a). Accordingly, Lowry and Zucker (1991) attempted to assess the presence of separation anxiety among a consecutive series of gender-referred boys. Lowry and Zucker transformed the DSM-III criteria for separation anxiety disorder into structured interview questions that could be answered by mothers as *Yes*, *Sometimes*, or *No*. This interview schedule was administered to the mothers of 47 gender-referred boys (mean age, 6.2 years). A "conservative" diagnosis of separation anxiety disorder was given if the mother answered *Yes* to questions in three of nine content domains, as is required in DSM-III. A "liberal" diagnosis of separation anxiety disorder was given if the mother answered *Sometimes* or *Yes* to questions in three of nine content domains. Interscorer reliability was available for the majority of protocols from audiotape. Without resolution, interscorer agreement exceeded 95% for specific questions and was 100% for final diagnostic decisions.

Of the 47 boys, 29 (61.7%) were judged to meet the complete DSM-III-R diagnostic criteria for gender identity disorder; the remaining 18 boys (38.3%) all showed signs of gender identity disorder, but did not meet the complete criteria. Using the conservative criteria, there was no relation between the presence of gender identity disorder and separation anxiety disorder; however, there was a relation between the two diagnoses when the liberal definition for the latter diagnosis was used. Of the 29 boys who met the complete criteria for gender identity disorder, 16 (55.1%) met the criteria for separation anxiety disorder compared to only 4 of the 18 boys (22.2%) who did not meet the complete criteria for gender identity disorder ( $\chi^2 = 3.7$ ,  $p < 0.05$ , one-tailed).

Other analyses showed that the boys who met the criteria for separation anxiety disorder were comparable with the boys who did not with regard to age, IQ and parent's social class, but were more likely to come from a mother only or "reconstituted" family. For the entire group, the number of separation anxiety traits scored as *Sometimes* or *Yes* correlated significantly, albeit modestly, with the CBCL Internalizing T score ( $r = 0.303$ ), but not with the CBCL Externalizing T score ( $r = 0.208$ ).

Although these results do not clarify the causal relation between separation anxiety and gender identity disorder, they provide some support for the notion that boys with gender identity problems manifest difficulties in the area of separation anxiety and, perhaps more generally, in affect regulation.

The possibility that boys with gender identity disorder are prone to anxiety and perhaps more specifically, separation anxiety, suggests a linkage between

attachment relations with mother and psychosexuality. Normative separation anxiety peaks at about 18 months (Kotelchuck, Zelazo, Kagan & Spelke, 1975), a time that is not too distant from the developmental period in which the signs of both typical and atypical gender development first appear. It is also known that anxious or insecure attachments, as conceptualized and measured by proponents of ethological attachment theory (e.g. Ainsworth, Blehar, Waters & Wall, 1978), can be assessed as early as 12 months of age. Thus, it is conceivable that a temporal model connecting anxious attachment and gender identity disorder could be studied longitudinally. The rarity of frank gender identity disorder would make this an expensive and almost impossible task to carry out; however, recent advances in the assessment of attachment relations during the preschool years (Bretherton & Waters, 1985; Greenberg, Cicchetti & Cummings, 1990) suggest a more plausible preliminary strategy. Given that many boys with gender identity disorder are referred during the preschool years, it would be of interest to assess formally the quality of their attachment relation to mother. If one were to demonstrate a high rate of anxious (insecure) attachment to the mother in boys with gender identity disorder, it would certainly call for a more comprehensive investigation regarding how these two important domains of functioning are linked.

### Long-Term Follow-Up

Green (1987) has conducted the most extensive long-term follow-up boys with gender identity disorder. This study can be used as a benchmark for the other published follow-up reports (Bakwin, 1968; Davenport, 1986; Kosky, 1987; Lebovitz, 1972; Money & Russo, 1979; Zuger, 1978, 1984). At the moment, insufficient numbers of girls have been followed prospectively to draw provisional conclusions about long-term outcome.

Green's (1987) study contained an original sample of 66 feminine and 56 control boys assessed initially at a mean age of 7.1 years (range, 4–12). About two-thirds of the boys in each group were reevaluated for a final time at a mean age of 18.9 years (range, 14–24). A semistructured clinical interview was employed to assess sexual orientation in fantasy and behaviour on Kinsey et al.'s (1948, pp. 636–641) 7-point sexual orientation continuum, where 0 equals exclusive heterosexuality and 6 equals exclusive homosexuality. Table 83.4 summarizes Green's follow-up data. Depending on the measure (fantasy or behaviour), 75–80% of the previously feminine boys were either bisexual or homosexual at follow-up versus 0–4% of the control boys.

A word is in order regarding the combining of Green's bisexual and homosexual outcomes. It is com-

**Table 83.4**

Sexual orientation in fantasy and behaviour at follow-up

Kinsey Rating	Feminine Boys ( <i>N</i> = 44)	Control Boys ( <i>N</i> = 35)
<i>Bisexual/Homosexual<sup>a</sup></i>		
Fantasy	33 (75%)	0 (0%)
Behaviour	24 (80%)	1 (4%)
<i>Heterosexual<sup>b</sup></i>		
Fantasy	11 (25%)	35 (100%)
Behaviour	6 (20%)	24 (96%)

*Note.* Data from Green (1987). Not all subjects had interpersonal sexual experience, so the *N* is smaller for this rating.

<sup>a</sup>Kinsey rating from 2 to 6.

<sup>b</sup>Kinsey rating from 0 to 1.

mon for homosexual men to recall bisexual behaviour during adolescence; thus, it is likely that those adolescent subjects who described themselves as bisexual will move, with time, toward a more exclusively homosexual orientation. Some authors argue that most men with bisexual behaviour are invariably preferentially homosexual as judged, for example, by penile plethysmography (see, e.g., Freund, 1977; Langevin, 1983).

Green also reported on the gender identity status of the 44 previously feminine boys. He indicated that one youngster, at the age of 18 years, was gender-dysphoric to the extent of considering sex-reassignment surgery.

Green's data compare favourably with the other six follow-up reports, none of which employed a comparison group. Combining these reports, there were 55 boys seen in follow-up, usually in late adolescence or young adulthood (range, 13–36 years). (Excluded from these reports were cases first assessed in adolescence and then later followed-up [for details, see Zucker, 1985, 1990b].)

At follow-up, five were classified as transsexual, 21 were classified as homosexual, one was classified as a (heterosexual) transvestite, 14 were classified as heterosexual, and 14 could not be rated with regard to sexual orientation. Excluding these last 14 cases, then 27 of 41 boys (66%) had atypical (i.e., transsexual, homosexual or transvestite) outcomes.

The results of these prospective studies are consistent with those of retrospective studies of adult homosexuals, which have repeatedly shown that homosexual men and women recall more cross-gender behaviour in childhood than heterosexual men and women (e.g., Bell et al., 1981; Bieber et al., 1962; Harry, 1982; Saghir & Robins, 1973; Whitam & Mathy, 1986). Thus, there is now sufficient evidence, from both retrospective and prospective data sets, to conclude that patterns of childhood gender identity are strongly associated with patterns of later sexual orientation, which represent one of the more powerful forms of continuity to emerge from research in developmental psychiatry over the past 30 years (cf. Zucker, 1987).

Recall interviews with adult transsexuals with a "homosexual" sexual orientation (cf. APA, 1987; Blanchard et al., 1987) almost invariably document a childhood cross-gender history. The prospective studies of children with gender identity disorder, however, have yielded only a handful of transsexual outcomes. Moreover, only one of these patients actually received sex-reassignment surgery (Lebovitz, 1972), and this patient was atypical in that he had Klinefelter's syndrome. Thus, it is not yet clear whether the "transsexual" patients identified in the prospective studies will persist in their desire to change sex and actually receive hormonal and surgical sex reassignment. At present, then, the agreement between prospective and retrospective studies is far less for transsexualism than for homosexuality.

Where have all the transsexuals gone? There are at least three possibilities. First, as Weinrich (1985) has argued, the reason may be a simple statistical one. Because the base rate of transsexualism is so low, even within the population of cross-gender-identified children, large sample sizes would be required to "scoop in" the few transsexual patients. A second possibility concerns referral bias. It is conceivable that transsexuals grow up in families in which the cross-gender behaviour is never experienced as "dystonic" hence, a clinical assessment is not sought (see Green, 1974). Thus, clinic-referred samples may not perfectly reflect the universe of children with gender identity disorder. Lastly, the natural history of cross-gender identification may be altered by the assessment process itself and by therapy when this occurs. Reductions in cross-gender identity during childhood may well lower the risk for subsequent transsexualism.

### Etiological Models

In contemporary sexology, little is settled regarding the determinants of gender identity, gender role, and sexual orientation. There are advocates of predominantly biological, psychological, and sociological influences. Others argue for integrative analyses, such as the currently popular "biopsychosocial" perspective. In this section, we review some of the major etiological, or quasi-etiological, studies of the gender identity disorder of childhood and "allied" conditions, including transsexualism, homosexuality, and certain forms of hermaphroditism.

### Biological Models

The search for biological correlates or determinants of psychosexuality in humans has been a slow, complex process, with many leads coming to dead ends. Several years ago, for example, it was claimed that the H-Y antigen status of transsexuals was "inverted" (e.g.,

Eicher et al., 1979a, b; 1981). There was a partial replication by Engel, Pfafflin and Wiedeking (1980), but subsequent attempts were non-confirmatory (Ciccarese, Massari & Guanti, 1982; Wachtel et al., 1986). As noted by Hoenig (1981, 1985, pp. 53–61), more stringent testing conditions by the original research team resulted in a failure to replicate and there were high rates of false positives. Research in this area has since ceased. Nevertheless, the study of other biological variables remains a central endeavour in contemporary sexological research.

**Genetics** Genetic influences on the gender identity disorder of childhood have not been well studied. Clinical data from several centres have yielded virtually no concordance among non-twin siblings (e.g., Green, 1974, 1987; Zucker et al., 1985). Case reports of both monozygotic (MZ) (Chazan, in press; Green & Stoller, 1971) and dizygotic (DZ) twins (Esman, 1970; Zucker, Bradley & Hughes, 1987) have all been discordant for gender identity disorder. Zuger (1989) reported an unremarkable prevalence of homosexuality in the first or second degree male (4%) and female (1%) relatives of 55 child and adolescent males with gender identity disorder.

Familiality for transsexualism has also not been well studied. Case reports have documented the occurrence of concordance among twins and non-twin siblings (reviewed in Hoenig, 1985; see also Joyce & Ding, 1985), but these studies are unable to separate genetic from environmental influences.

Four decades ago, Kallmann (1952a, b) reported a 100% concordance rate for homosexuality among MZ twin males compared to a 15.4% concordance rate for DZ twin males. Since then, there has been a sprinkling of case reports in the English language literature of MZ twins reared together, some finding concordance for homosexuality, others finding discordance. Among the discordant male pairs, some reports noted that the homosexual twin had shown behavioural signs of femininity during childhood (e.g., Friedman, Wollesen & Tandler, 1976; McConaghy & Blaszczyński, 1980; Zuger, 1976). The sporadic nature of the case report literature, the existence of discordant cases, methodological criticisms of Kallman's data (Rosenthal, 1979, pp. 250–255), and an antipathy towards genetic research in the decades after World War II (see Rosenthal, 1970) all seemed to reduce interest in the potential contribution of genetics to sexual orientation development and, indirectly, gender identity.

Recent studies, however, have sparked renewed attention to genetic factors. Eckert, Bouchard, Bohlen and Heston (1986) described two pairs of MZ twin males reared *apart*: one pair of male twins was primarily discordant for homosexuality, but the other pair was

concordant and, when the twins were reunited in adulthood, became sexual partners! Four female MZ twin pairs, also reared apart, were all discordant for homosexuality. Using substantially larger samples of male twins (reared together), Bailey and Pillard (in press) and Buhrich, Bailey and Martin (1991) have shown significantly greater concordance for homosexuality among MZ than DZ twins. Coupled with recent evidence for an elevated incidence of homosexuality in the brothers of homosexual men and the sisters of homosexual women (Bailey, Benishay & Pyron, 1991; Bailey, Willerman & Parks, 1991; Pillard, 1990; Pillard, Poumadere & Carretta, 1981, 1982; Pillard & Weinrich, 1986; cf. Blanchard & Sheridan, 1992), the role of genetic factors in sexual orientation development requires a fresh examination. The quantitative genetic modelling techniques employed by Buhrich et al. (1991), Bailey and Pillard (in press), and Mitchell, Baker and Jacklin (1989) show great promise in this regard. Assuming that replication research verifies the role of inheritance, it still remains to be determined what the mechanism of transmission will be. On this point, other work will be required.

**Prenatal Hormones** The effects of prenatal patterns of sex steroid secretion on the development of sex dimorphic behavioural development have been studied for some time now. Scores of animal studies have shown an important role for hormonal influences on sex dimorphic behaviour (e.g., Beach, 1975; Goy & McEwen, 1980; Money & Ehrhardt, 1972). Although there is both within- and cross-species variations, the basic principle that the prenatal hormonal milieu shapes or induces a predisposition for certain sex-dimorphic behavioural patterns has marshalled a great deal of support.

The effects of prenatal sex hormones on human psychosexual development are, no doubt, less powerful than they are in lower animals (for some recent reviews, see Ehrhardt & Meyer-Bahlburg, 1981; Gladue, 1987, 1988, 1990; Gooren, 1988, 1990; Gooren Fliers & Gourtney, 1990; Hines, 1982; Meyer-Bahlburg, 1984b, Money, 1987, 1988). Perhaps the main bone of contention is whether variations in the prenatal hormonal milieu have any effect at all and, if they do, are of practical significance. On this point, there is often intense, acrimonious commentary (e.g., Bleier, 1984; De Cecco, 1987; Doell, 1990; Rogers & Walsh, 1982; Schmidt, 1984).

The syndrome of congenital adrenal hyperplasia (CAH) nicely illuminates the complexities of hormone-behaviour relations. CAH is an inherited, autosomal recessive disorder of adrenal steroidogenesis. Among Caucasians, the incidence is about one in 5000–15 000 live births (White, New & Dupont, 1987). Due to the

excessive adrenal androgens, girls with this disorder are born with ambiguous or fully masculinized external genitalia. Surgical repair can normalize the appearance of the external genitalia and cortisone-replacement therapy, available since 1950, normalizes the malfunctioning endocrine system and, in theory, essentially shuts down the excessive production of adrenal androgen (Winter, 1980).

Treated CAH, then, has been used as a model “experiment of nature” in which the effects of abnormal prenatal hormone exposure on postnatal sex-dimorphic behaviour can be observed. Initial studies indicated that girls with CAH were more masculine and/or less feminine than were control girls in their gender role behaviour (Ehrhardt & Baker, 1974; Ehrhardt, Epstein & Money, 1968), a finding that has been recently replicated by others (e.g., Berenbaum & Hines, in press; Dittmann, 1989; Dittman et al., 1990a, b). The core sense of self as female, however, seems to be less affected, with very few girls with CAH showing signs of a formal gender identity disorder, although the prevalence of gender dysphoria is probably higher than would be expected in the general population (cf. Money, 1968; Zucker et al., 1987). Follow-up studies have indicated higher rates of bisexuality/homosexuality and lower rates of marriage and sociosexual experience of any kind in young adult women with CAH (Money, Schwartz & Lewis, 1984; Mulai-kal, Migeon & Rock, 1987).

The CAH human studies suggest a continuity with research on lower animals, in which experimental manipulation of the prenatal hormonal milieu affects the patterning of sex-dimorphic behaviour. Critics of the biological interpretation of the CAH data have appealed to alternative explanations—parental response to the ambiguous genitalia, expectancy effects for a child with an ambiguous sex, medication side effects, and so on (e.g., Bleier, 1984; Huston, 1983; Quadagno, Briscoe & Quadagno, 1977). In fact, virtually no empirical work has assessed social influences directly, but there is some evidence that speaks against a social effects argument. For example, Berenbaum and Hines (in press; see also Berenbaum, 1990) found no relation between measures of physical masculinization (e.g., clitoral length) and degree of behavioural masculinity (cf. Dittman et al., 1990b). Observational and interview studies with parents of CAH girls need to be done to see if variations in their reactions to sex-typed behaviour augment or reduce the likely biologic predisposition toward culturally defined masculinity that their affected daughters possess; moreover, work needs to be done to understand better how CAH youngsters themselves understand their condition and what impact this has on their psychosexual development.

The CAH studies, as well as other conditions in which prenatal hormonal anomalies have been identified (for a recent review, see McCauley & Urquiza, 1988), implicate a role for hormonally-mediated influences on psychosexual development. As noted by Gladue (1988), however, individual differences in outcome suggest that "it is unlikely that the apparently direct and seemingly causal relationship between hormones and [central nervous system] development seen in animal models is directly applicable toward the human" (p. 402).

One remains in speculative terrain when it comes to a biological etiology for the gender identity disorder of childhood. Invariably, youngsters with this disorder show no signs of physical hermaphroditism, including hormonal anomaly. Recent studies with non-human primates, however, have shown that induction of "behavioural" hermaphroditism can occur without genital hermaphroditism (e.g., Goy, Bercovitch & McBair, 1988), suggesting that hormonal effects may influence brain-behavioural systems independently of physical genital differentiation. This is of particular interest, since several lines of research over the past two decades have quite tentatively implicated differences between heterosexuals and homosexuals on parameters that may have, in part, a prenatal hormonal basis, but for which there is clearly no impact on physical genital differentiation: patterns of adult luteinizing hormone secretion (Dorner, 1988; Dorner et al., 1975; Gladue, Green & Hellman, 1984), handedness (Lindesay, 1987; McCormick, Witelson & Kingstone, 1990; Tkachuk & Zucker, 1991), and spatial ability (Gladue, Beatty, Larson & Staton, 1990; Sanders & Ross-Field, 1987; Tkachuk & Zucker, 1991). Two recent studies of adult transsexuals have also reported a higher than expected prevalence of non-right-handedness (Gooren, 1991; Watson & Coren, 1991). Prevalence of non-right-handedness has not been evaluated in children with gender identity disorder, but boys with gender identity disorder, like homosexual men, perform more poorly on tests of spatial ability when compared to same-sex controls (Finegan, Zucker, Bradley & Doering, 1982; Grimshaw, Zucker, Bradley, Lowry & Mitchell, 1991). Further evidence for convergence between child and adult samples on these kinds of measures is clearly called for.

It is also possible that variations in the prenatal hormonal milieu within the "normal" range may exert predisposing effects on intrasex differences in postnatal sex dimorphic behaviour. Recent studies that have obtained prenatal hormone levels from amniotic fluid may provide a methodological tool to examine hormone-behaviour relations in normative populations (e.g., Finegan, Bartleman & Wong, 1988). Consider, for example, rough-and-tumble play and activity level

in boys. Boys with gender identity disorder usually dislike rough-and-tumble play (Green, 1976, 1987) and appear to have a relatively low activity level (Zucker & Bradley, 1988). Both behaviours show a strong sex dimorphism (DiPietro, 1981; Eaton & Enns, 1986). It is likely that the two behaviours, which are probably closely related, are at least partly determined by biological factors (cf. Meaney, Stewart & Beatty, 1985).

As noted earlier, boys with gender identity disorder also seem more prone to internalizing behavioural difficulties (Zucker, 1990a). A very anxious boy may also be likely to be timid and thus to avoid rough-and-tumble play. Such a boy may avoid other boys as playmates, feel more comfortable in the presence of girls, and identify more with his mother than with his father. Reactions to his anxiety and timidity may exacerbate or attenuate them; for example, exposure to less active boys may accustom the anxious boy to male peers, whereas exposure to only female peers and their conventionally feminine activities may aggravate a predisposition to cross-gender identification. Thus, the boy may eventually decide that it is better to be a girl than a boy (Green, 1987).

**Maternal Stress** In rats, the exogenous manipulation of maternal stress by aversive means during a specific period of the pregnancy has been shown to alter fetal testicular enzyme activity, resulting in a temporary androgen deficiency. In turn, this appears to have an anomalous effect on postnatal sex-dimorphic behaviour in male offspring, including demasculinized (e.g., reduced initiation of copulation) and feminized (e.g. lordosis) sexual behaviour, although reproductive morphological structures remain intact (Ward, 1984).

This experimental procedure, dubbed the "prenatal stress syndrome", led Dorner et al. (1980) to predict that the mothers of homosexual men would have experienced more prenatal stress than the mothers of heterosexual men. Dorner et al. (1980) inferred this on the basis of data showing that, in the eastern part of Germany (formerly the German Democratic Republic), significantly more homosexual men were born during World War II and the early postwar years (1941–1947)—a period of presumed exogenous stress for pregnant women—than in the years before or after the war. In a second study, Dorner, Schenk, Schmiedel and Ahrens (1983) asked 100 bisexual/homosexual men and 100 heterosexual men about maternal stressful events that may have occurred during their own prenatal life. Subjects were asked to consult their parents for this information. It was reported that the bisexual and homosexual men had mothers who experienced more "moderate" and "severe" stress during the pregnancy than did the mothers of heterosexual men.

These studies have been the subject of various methodological criticisms (see, e.g. Bailey, 1989), including absence of reliability checks for both the independent (maternal stress) and dependent (sexual orientation) variables, demand characteristics, and alternative interpretations (e.g., confounds with father-absence). Moreover, it should be recognized that the presumed mechanism of influence—induction of temporary androgen deficiency in the human male fetus—has not been directly documented.

Mechanisms of influence aside, there have been several efforts to replicate the alleged association between recalled maternal stress and sexual orientation. Ellis, Ames, Peckham and Burke (1988) asked mothers of heterosexual, bisexual, and homosexual men, and mothers of heterosexual and homosexual women to complete a questionnaire that included information on the number and severity of stressful life events during the year prior to and during the proband's pregnancy. Because the second trimester of humans is considered a sensitive period for sexual differentiation of the brain (Money, 1988), stress during the pregnancy was rated separately by trimester. Ellis et al. (1988) found that the mothers of homosexual men had a higher stress severity score during the year *prior* to the pregnancy, but not during it, compared to the mothers of bisexual and heterosexual men. When analysed by trimester, it was found that the mothers of homosexual men recalled more severe stress 9–12 months *before* the pregnancy and during the second trimester compared to the mothers of heterosexual men. Mothers of heterosexual and homosexual women did not differ significantly in their recollection of stress.

Unfortunately, Ellis et al.'s (1988) statistical analyses were flawed, including an inflated Type I error rate. Descriptively, the most consistent aspect of the Ellis et al. data was that the mothers of homosexual men recalled more severe stress than did the mothers of heterosexual men both *before* and *during* the pregnancy up until the third trimester, when the pattern of means inverted. These data do not appear to give strong support to the selective importance of stress during the second trimester, although the pattern of results does not rule out that stress during the second trimester may have had its hypothesized effect on the fetus.

Perhaps the methodologically tightest study on the maternal stress–sexual orientation hypothesis has been conducted by Bailey et al. (1991; see also Bailey, 1989). In this study, recalled maternal stress during each of the three trimesters correlated close to zero with Kinsey ratings of sexual orientation in fantasy and self- and maternal-report measures of childhood cross-gender behaviour in heterosexual and homosexual men (absolute *rs* ranged from 0.00 to 0.11). Interestingly, however, Bailey et al. (1991) found that maternal

“stress-proneness” was modestly correlated with degree of recalled boyhood effeminacy (by both the men themselves and the mothers), which bears some consistency with the interpretation offered above regarding the Ellis et al. (1988) data set. Similar null findings have been reported by Wille, Borchers and Schultz (1987) and Schmidt and Clement (1990), although these two studies were less solid methodologically than the Bailey et al. (1991) report. On the whole, however, this particular approach to assessing the impact of maternal stress on parameters of psychosexual development does not seem to support the basic hypothesis derived from work with rats. Perhaps more sensitive assessments of human maternal stress (cf. Levin & DeFrank, 1988) conducted in a prospective, longitudinal design will bear more fruit.

**Physical Attractiveness** Variations in physical attractiveness are determined, no doubt, at least partially by objective, biophysical properties (Langlois & Roggman, 1990). By way of serendipity, (facial) physical attractiveness was implicated as an etiological factor in one of the first, small-scale clinical studies of boys with gender identity disorder (Stoller, 1968a). Maternal report of the physical beauty of their feminine sons during infancy led Stoller (1975) to remark that “We have noticed that they often have pretty faces, with fine hair, lovely complexions, graceful movements, and—especially—big, piercing, liquid eyes” (p. 43). Although Stoller (1975) placed great weight on parental influences, it was also suggested that the extreme physical attractiveness of the boy served as a type of stimulus—the “spark”—that facilitated parental feminization, particularly on the mother's part.

Green (1987) and his colleagues (Green, Williams & Goodman, 1985; Roberts et al., 1987) systematically studied physical attractiveness in a larger sample of feminine boys and a male control group. At the time of assessment (*M* age, 7.1 years), the parents of both groups of boys were asked to describe the faces of their infant sons. Blind ratings of interview transcripts and questionnaire responses showed that the parents of the feminine boys more often described their sons during infancy as “beautiful” and “feminine” than did the parents of the controls. There was also a trend for the parents of the feminine boys to recall that strangers commented, “He would make a beautiful girl,” than did the parents of the control boys.

In general, Green's (1987) data lend some support for Stoller's (1968a, 1975) clinical observations. Nevertheless, it remains unclear the extent to which Green's data implicate objective properties of the feminine boy as infant or parental retrospective distortions. It could be argued that parental recall of earlier attractiveness was affected by the son's current femininity. Recollection

tion of the boy as feminine would provide a certain continuity to, or perhaps even an explanation for, the current behavioural pattern. It is also possible that parents who condoned or reinforced the boy's femininity (Green, 1987) might be particularly prone to distort memories in the direction of a more feminine-like infant.

Zucker, Wild, Bradley and Lowry (in press) compared the attractiveness of 17 boys with gender identity disorder to that of demographically matched clinical control boys. Facial and upper-torso photographs were taken at the time of clinical assessment (mean age, 8.1 years). College students, blind to group status, provided ratings with regard to five traits: attractive, beautiful, cute, handsome and pretty. With the exception of the trait "handsome," the other traits were intended to be somewhat "feminine" in valence, in order to be consistent with the clinical accounts of the physical appearance of boys with gender identity disorder (e.g., Green, 1974; Stoller, 1968a). It was found that the boys with gender identity disorder were rated as significantly more attractive on all five traits than were the clinical control boys.

These data therefore complemented and extended the parental recall data reported by Green and his colleagues. It remains unclear, however, the extent to which the between groups difference in attractiveness was due to objective, physical differences, socially-shaped differences, or some proportional combination of both. It is conceivable, for example, that a structural analysis of the boys' faces would yield differences in the direction suggested by Hildebrandt and Fitzgerald (1979) to be associated with cuteness in infants. Along the same lines, it would appear warranted to analyse infant photographs of boys with gender identity disorder to see if similar properties could be identified. This would be particularly intriguing from a psychosexual perspective, since recent studies have implicated objective properties of infant faces, even newborns, that are correlated with accurate predictions of biological sex (e.g., Gewirtz & Hernandez, 1984, 1985; Gewirtz, Weber & Nogueras, 1990).

Regardless of the role of objective facial properties in determining attractiveness, clinical evidence suggests that the parents of some feminine boys, particularly the mothers, will dress and style the hair of their sons in a manner that might be construed as "cute" or unmasculine, possibly even feminine (e.g., Green, 1974). Clinical experience also suggests that the boys themselves will shape their appearance to create a softer, cuter look. This type of evidence would implicate social (subjective) determinants to the attractiveness. When engineered by the boys, the heightened feminine-like attractiveness could even be interpreted as simply a symptom of the underlying cross-gender identification.

Correlational data reported by Zucker et al. (in press) on the relation between age and attractiveness appeared to implicate social influences, at least to some extent. Age and attractiveness were substantially negatively correlated among the clinical control boys, suggesting that with age these boys were losing the features that elicited higher feminine-valenced attractiveness ratings. In contrast, the relation between these two variables was substantially lower in the gender identity disorder group, suggesting that, with age, these boys retained the features that elicited the female-valenced attractiveness ratings.

### Psychosocial Models

In the section on associated psychopathology, some consideration was given to the putative role of non-specific parental/familial psychopathology in the genesis of gender identity disorder. In this section, consideration is given to psychological variables that have been viewed as of specific relevance in influencing individual differences in sex dimorphic behavior.

**Social Reinforcement** Since Mischel's (1966) seminal, largely theoretical essay 25 years ago, a reinforcement account of sex-typing has been given much attention. In some respects, social shaping of sex-typed behaviour has great intuitive appeal, perhaps because of its common sense, parsimonious nature. Yet, it has been repeatedly pointed out that empirical confirmation of social reinforcement explanations of sex-typing has been slow in coming, a view that was advanced most forcefully in Maccoby and Jacklin's (1974) *tour de force* of the sex difference literature.

Although a comprehensive review of this literature is beyond the scope of this article, several overarching questions can be considered. What is the evidence that parents (and others), on average, respond in sex-specific ways to sex-typed behaviour in boys and girls? Are there specific domains of behaviour that are responded to more than others? Are there particular age periods in which differential parental responses occur? Do parents (and others) actually shape sex dimorphic behaviour or do they simply respond to differences between boys and girls that are already present? Do parental responses actually affect the subsequent behaviour of the child?

Because the behavioural signs of gender identity disorder first appear in the toddler and preschool years, normative research in this age range has the most important bearing on etiological issues. Such behaviours most typically include toy, dress-up, and role play; thus, parental responses in this domain are of particular import.

Work by Fagot and colleagues has focused on the microsocial parent-child interactions vis-à-vis these

kinds of sex-dimorphic behaviours. For example, Fagot (1978) conducted home observations of 24 boy and girl toddlers (mean age, 22.8 months) and categorized parental responses as positive, neutral, or negative to specific child behaviours that were sex-preferred, i.e., engaged in significantly more frequently by boys or by girls (e.g., boys: use of transportation toys; play with blocks; girls: play with dolls, soft toys; dress up). Of seven sex-preferred behaviours, parents responded differentially as a function of the child's sex to four of them. For example, block play was responded to more positively when engaged in by boys than by girls whereas doll play was responded to more positively when engaged in by girls and more negatively when engaged in by boys (cf. Caldera, Huston & O'Brien, 1989; Eisenberg, Wolchik, Hernandez & Pasternack, 1985; Jakhlin, DiPietro & Maccoby, 1984).

Fagot's (1978) study (see also Fagot & Hagan, 1991) did not, however, address two key questions. Were the behavioural sex differences present prior to the initiation of differential parental reactions or were they shaped by them (cf. Snow, Jacklin & Maccoby, 1983)? Do parental reactions affect the subsequent behaviour of the child?

Fagot and Leinbach (1989) have provided evidence that parental attention to (putatively) sex-dimorphic behaviour affects the pace at which children develop patterns of sex-differentiated behaviour. Toddlers and their parents were studied at 18 and 27 months. At 18 months, none of the toddlers were able to "pass" a gender-labelling task (Leinbach & Fagot, 1986), but at 27 months there was more variation: 48% passed ("early labellers") and 52% failed ("late labellers"). At 18 months, future early and late labellers did not differ in their degree of sex-typing; in fact, both groups showed little evidence of sex-differentiated behaviour. At 27 months, however, early labellers showed stronger patterns of sex-differentiated behaviour than did late labellers; for example, boys who were early labellers played more with masculine toys than did the other three groups whereas girls who were early labellers played more with feminine toys than did the other three groups (cf. Fagot et al., 1986).

Based on parent-child observation at 18 months, it was found that parents of future early labellers were more likely to emit *both* positive and negative responses to masculine *and* feminine behaviour in their toddlers than were the parents of late labellers; however, the two groups of parents did not differ in their rates of "instructional" behaviour (e.g., directive, verbal interaction) in response to their toddler's sex-typed play.

It is clear that these findings do not provide unequivocal support for the social learning account of sex-

typing, since early labellers received more positive *and* negative feedback about their masculine and feminine behaviour than did late labellers. This led Fagot and Leinbach (1989) to emphasize the importance of affect per se in sensitizing toddlers to the salience of gender. The scenario, however, gets complicated because by 27 months parents of both groups were much more likely to give their toddlers positive feedback for same-sex play than for cross-sex play and more negative feedback for cross-sex play than for same-sex play (except for fathers of girls). By age 4, both groups of toddlers showed similarly conventional sex role preference scores although the previously early labellers had a more sharply developed awareness of sex role stereotypes than did the previously late labellers.

Do studies of this genre contribute to our understanding of parental responses to the early sex-dimorphic behaviour of children with gender identity disorder? Clinical experience from diverse quarters examining children with gender identity disorder has suggested that parental responses to early cross-gender behaviour are typically that of neutrality (tolerance) or of positive encouragement. Parental recall of such responses taken from clinical and structured interviews have been provided by Green (1974, 1987). Based on seminal observations of boys with gender identity disorder, Green (1974) provisionally concluded that "what comes closest so far to being a *necessary* variable is that, as any feminine behavior begins to emerge, there is *no* discouragement of that behavior by the child's principal caretaker" (p. 238, emphasis in original). In a subsequent report (Roberts et al., 1987), ratings of recalled parental reactions to the boy's initial feminine behaviours were shown, on average, to be in the neutral to positive range. For the mothers, initial approval of feminine behaviours was significantly correlated with a composite measure of the boy's femininity at the time of the actual assessment (see also Green, 1987). Green's (1987; Roberts et al., 1987) empirical study appears to support the proposition that parental tolerance or encouragement of nascent feminine behaviour is part of the etiological puzzle.

It seems, therefore, that there is some evidence of continuity between normative and clinical studies of reinforcement (or tolerance) of certain core sex-typed behaviours. Lytton and Romney's (1991) recent meta-analysis of parental gender socialization in different behavioural domains led them to conclude that, with one exception, there was "little differential socialization for social behaviour or abilities". The exception was in the domain of "encouragement of sex-typed activities and perceptions of sex-stereotyped characteristics", for which the mean effect sizes for mothers, fathers and parents combined were 0.34, 0.49, and

0.43, respectively. Although Lytton and Romney's (1991) overall conclusion minimized the influence of parental socialization on sex-dimorphic behaviour, the domain for which clear parental gender socialization effects were found is precisely the area that encompasses many of the initial behavioural features of gender identity disorder.

Although social reinforcement appears to be an important mechanism in explaining the genesis of individual differences in sex dimorphic behaviour, it would be prudent to note the limitations in the data sets currently available, particularly as they pertain to understanding children with gender identity disorder. First, it is somewhat difficult to know how parents of "normal" children react to cross-gender behaviours because the base rates seem to be so low. For example, in the Fagot (1978) study, toddler boys engaged in (female) dress-up play so infrequently that statistical comparisons with girls who engaged in such behaviour could not be made! Cross-dressing is one of the first behavioural signs of gender identity disorder (Green, 1976), often appearing between the ages of two and four, so it is somewhat frustrating to note how little is known about how such a behaviour might be responded to in normative populations.

At least under conditions of observation, parents seem to give more positive attention to same-sex behaviours than they give negative attention to cross-sex behaviours (Fagot & Leinbach, 1989); thus, future studies of children with gender identity disorder may try to assess encouragement of same-sex behaviour separately from the discouragement of cross-sex behaviour (cf. Green, 1987). When instructed to encourage masculine behaviour, mothers of boys with gender identity disorder were as adept as control mothers, although they were less likely to encourage such behaviour during a no-instruction condition (Doering, 1981).

Second, in normative populations the meaning of a positive parental response to a rare behaviour is difficult to interpret (cf. Langlois & Downs, 1980). Roberts et al. (1987) encountered a similar problem in that their control group of boys so rarely engaged in feminine behaviours that it was impossible to generate ratings of parental reactions. Thus, how parents of "normal" children react to consistently deviant sex-typed behaviour is not an easy task. Creation of laboratory simulations is a possible solution (see Langlois & Downs, 1980), but ecologically suspect. Perhaps interview studies that attempt to understand the importance and meaning parents give to gender development would be of help (cf. Antill, 1987; Brooks-Gunn, 1986).

Third, although variations in parental behaviour may affect the child's sex-typed behaviour, the overall pattern of sex-typed behaviour is likely to still be with-

in conventional range. This invokes, then, a crucial assumption of developmental psychopathology, namely that one is simply dealing with variations on a spectrum of normality and deviance. In the case of gender identity disorder, the behaviour pattern is often so extreme, one would want to look for extreme environmental responses, such as persistent parental tolerance of cross-gender behaviour, marked parental ambivalence regarding the child's sex, and even active promotion of cross-gender behaviour. In some instances, the clinical literature provides such evidence (e.g., Lothstein, 1988; Stoller, 1975, ch. 17; Zucker & Ipp, 1990). Green (1974) reported that about 15% of the mothers in his sample periodically dressed their sons in "girls' clothes" during infancy and toddlerhood, 8% were occasionally cross-dressed by female siblings, and 10% were subject to such behaviour by grandmothers. It is clear that the final word has not been written about the role of early reinforcement patterns, but important lines of inquiry have been opened, particularly as a result of the detailed microsocial observation systems devised by developmentalists to describe the genesis of parent-child sex-typed interactions.

**Parental Identifications** Boys with gender identity disorder appear to be closer emotionally to their mothers than to their fathers (Green, 1974, 1987; Sherman, 1985; Zuger, 1970). This relationship pattern can easily be conceptualized in three ways: a parent to child effect, a child to parent effect, or a reciprocal influence/transactional effect. Adherents of psychosocial etiology favour the first interpretation (e.g., Stoller, 1968c, 1979) whereas adherents of a constitutional etiology favour the second (e.g., Isay, 1987; Zuger, 1970, 1980). Perhaps etiological fencesitters favour the third!

The concept of "parental identification" was particularly influential in guiding early social learning studies of normative gender development (e.g., Sears, Rau & Alpert, 1965) that emphasized parent to child effects. To some extent, experience with clinical cases appeared to highlight the alleged phenomenon. For example, in Stoller's (1968a, 1975, 1979) clinical studies of boys with gender identity disorder, he described an overly close relationship between mother and son and a distant, peripheral father-son relationship. Stoller (1985a) has held that such qualities are of etiological relevance: "The more mother and the less father, the more femininity" (p. 25).

Green (1987; see also Green et al., 1985) attempted to quantify the amount of shared time between parents of feminine boys and controls during the first 5 years of life. The fathers of feminine boys recalled spending less time with their sons from the second to fifth year than did the fathers of controls. Recalled father-son shared

time had important correlates; for example, less shared time was associated with more femininity in the boys as well as a more homosexual orientation at follow-up (Green, 1987). In contradiction to the over-closeness hypothesis, the mothers of feminine boys also reported spending less time with their sons than did the mothers of controls.

Green's (1987) data on father-son shared time were consistent with a large body of clinical literature whereas the mother-son data were not. Negative findings are difficult to interpret, particularly when they have not been consistently replicated. In this instance, they are difficult to reconcile with data showing that feminine boys feel closer to their mothers than to their fathers (Green 1987). Perhaps qualitative features of the mother-son relationship, such as attunement to each other's feelings, would have been a more sensitive index of the dyad's nature. It is also possible that relative time spent with mother vs father would have yielded differences between Green's feminine boys and the controls.

**Maternal Psychosexual Development** Based on clinical data, Stoller (1986a) reported that mothers of very feminine boys had childhood psychosexual conflicts. Although initially feminine, he argued that "a degree of masculinity beyond what usually [would] be called tomboyishness" (p. 298) developed following a breach in the father-daughter relationship. A desire to be a male was relinquished at puberty, yet sociosexual experience was minimal and, as adults, these women married distant men with whom sexual relations were poor. These women appeared to be uncomfortable with their femininity: "While these women have a feminine quality, inextricably woven in is this other, difficult to describe but easy to observe use of certain boyish or 'neuter' external features" (Stoller 1968a, p. 298).

Green's (1987) effort to verify Stoller's observations yielded mixed results. Mothers of feminine boys were more likely than the mothers of the control boys to describe themselves as tomboys (Green et al., 1985), but they did not differ with regard to their recall of specific sex-typed behaviours (Green 1987, p. 68). The two groups of mothers also did not differ with regard to the extent of adolescent sociosexual experience. Finally, Green's (1987) trait assessment of childhood masculinity-femininity did not provide strong support for the possibility that less severe signs of cross-gender behaviour were present. From these data, it appears that mothers of very feminine boys, on average, do not have grossly atypical psychosexual histories. Perhaps other aspects of psychosexuality need to be studied, such as the mother's current attitude towards

men and her concurrent views regarding masculinity and femininity.

### Treatment

Treatment of children with gender identity disorder has been approached from diverse conceptual orientations, including behaviour therapy, psychotherapy, parent counselling, family therapy, group therapy and eclectic combinations (Green, 1974, 1987; Green, Newman & Stoller, 1972; Rekers, 1977, Rekers, Kilgus & Rosen, 1990; Zucker, 1985, 1990b; Zucker & Green, 1989; Zucker et al. 1985). It goes without saying that the theoretical lens through which one views psychopathology influences how treatment issues will be conceptualized. For example, proponents of behaviour therapy (e.g., Rekers 1977) have focused largely on the development of techniques to modify specific sex-typed behaviours, such as cross-dressing and exclusive play with opposite-sex toys, whereas psychodynamically oriented clinicians have a greater preference to view gender identity disorder in the context of family pathology and associated personality psychopathology in the child (Coates & Person 1985; Meyer & Dupkin 1985) and to place as much emphasis on treating these problems as the gender identity symptomatology itself. Thus, what (or whom) should be treated is embedded in the complexity of the theoretical frame by which the clinician attempts to understand the psychopathology.

The empirical evaluation of treatment efficacy for children with gender identity disorder is weak, much like the treatment efficacy for many types of childhood psychopathology (Kazdin, 1990). Comparative studies of different types of treatment approaches are simply not available; thus, any claim of superiority, particularly with regard to long-term effects, is unwarranted. Again, this is a problem that cuts across many areas of childhood psychopathology (Shaffer, 1984). There is virtually no systematic research concerning treatment in childhood and its effect on postpubertal outcome. The only long-term effect of treatment was reported on by Green (1987), who noted that the rate of bisexuality/homosexuality was similar in the boys who had received treatment in childhood compared to those who had not received treatment; however, Green's (1987) study was not primarily a treatment investigation, so this outcome should be viewed with caution. These caveats aside, several of the more salient treatment approaches will be discussed.

### Behaviour Therapy

Rekers (1977, 1985) and colleagues have provided the most systematic information regarding behav-

journal approaches. Using different types of behavioural techniques, such as differential social attention or reinforcement, token economy, and self-regulation, there is evidence that specific sex-typed behaviours can be either reduced or increased in their frequency. The techniques have been subject to two main limitations—stimulus specificity and response specificity. The first term refers to the phenomenon of a behaviour reappearing (e.g., cross-dressing) in the absence of the stimulus condition under which it was modified (e.g., parental negative sanctions, new environment). The second term means that the treatment did not generalize or influence untreated behaviours although they appear to be of the same type as the treated behaviour; thus, a procedure might modify cross-dressing, but not generalize to play with Barbie dolls, a behaviour that was not specifically subject to formal treatment techniques (for a review, see Zucker, 1985).

Despite these limitations, an overall analysis of the behaviour therapy case report literature suggests some impact on the presenting problem and apparently an impact on the child's overall sense of gender identity (Zucker, 1985). Rekers et al. (1990) recently provided group analysis of 29 boys treated by behaviour therapy techniques. At a mean follow-up of 51 months after treatment, it was found that "completion" of treatment (defined nominally) accounted for 20% of the variance in change scores, as defined by a reduction in ratings of cross-gender identification. Unfortunately, no published longer term follow-ups on this sample, in which their adolescent gender identity and sexual orientation were assessed, are available.

### Psychotherapy

The psychotherapy literature consists of a couple of dozen case reports (see Zucker, 1985, 1990c). Unlike the use of quantitative data in the behaviour therapy case report literature, the psychotherapy literature is much more descriptive and qualitative in nature (see, e.g. Schultz, 1979). Although difficult to quantify, the impression one gets from studying this literature is that many of the children had made gains by the end of their therapy. Because many of the parents of these children were in therapy and because in some instances the child received additional, concurrent treatments (e.g. as an inpatient), the precise mechanisms of change become even more difficult to disentangle. No systematic follow-up data on adolescent gender identity and sexual orientation are available from the psychotherapy case report literature (cf. Zucker, 1985).

Elsewhere, we have identified discernable themes in the psychotherapy case report literature (Zucker, 1985, 1990c; Zucker & Green, 1989). These include

an emphasis on the significance of the emergence of cross-gender behaviour during the preoedipal years, with concomitant attention to early object relations and general ego functioning. Attention is given to understanding the impact of the mother-child and father-child relationship on the formation of the gender symptomatology and the parental psychodynamics that putatively underlie their tolerance for the cross-gender behaviour. In a sense, then, this literature takes a more contextual approach in understanding the gender identity disorder than does the behaviour therapy literature.

### Conclusion

This review has covered several areas of research pertaining to gender identity disorder and allied conditions: epidemiology, diagnosis, assessment, associated psychopathology, follow-up, etiology and treatment. Over the past several decades, significant advances have been made in diagnosis and assessment such that the syndrome of the gender identity disorder of childhood can be properly evaluated by child clinicians. In contrast, advances in etiological research have been slower in coming although the increased sophistication in both biological and psychosocial methodologies are cause for optimism. Empirical evaluations of treatment have been particularly weak, in part because of the difficulties in conducting research on a relatively rare disorder. Perhaps the establishment of cross-centre collaborations will facilitate the filling of gaps in understanding this uncommon, yet intriguing, psychosexual condition of childhood.

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## Introduction

Transsexuality is an incongruence between sexual differentiation (determined by chromosomal, gonadal and hormonal sex) on the one hand and self-declared gender identity on the other. It is still highly controversial whether gender identity is (predominantly) determined by biological factors or by psychosocial gender assignment, particularly in the first two years of one's life (Naftolin & Butz, 1981; Erhardt & Meyer-Bahlburg, 1981; Meyer-Bahlburg, 1982). Money et al. (1955) defended the idea that gender identity was undifferentiated at birth and was formed by subsequent sex assignment and rearing in the first years of life, irrespective of internal and external somatic characteristics. This concept was challenged by Imperato-McGinley et al. (1979), who reported a successful reversal of gender identity of boys suffering from 5-alpha-reductase deficiency during puberty and concluded that androgen exposure has a more profound effect on gender identity than sex of rearing. This conclusion has been fundamentally criticized with good evidence (Meyer-Bahlburg, 1982). A wealth of information on sexual differentiation in mammals has emerged since the pioneering work of Alfred Jost: the X and Y chromosomes determine the nature of the gonad in the fetus, either ovary or testis. After gonadal determination, fetal endocrine function organizes the internal and external genitals along male lines in the presence of androgens or along female lines in the absence of androgens. Later gonadal secretions determine genital structure and function in the adult (Wilson et al., 1981). In some species gonadal androgen secretion at a critical period of intrauterine or neonatal life differentiates (future) central nervous system mechanisms (such as gonadotrophin control and sexual behavior). The metabolism of androgens to estrogens locally in the brain is a remarkable feature in sexual differentiation (MacLusky & Naftolin, 1981).

These differentiating effects of sex steroids are expressed in terms of changes in the central nervous system at the structural as well as the functional level,

resulting in dimorphic sexual behavior in adult animals. Administration of testosterone (T) to a perinatal female rodent initiates three events in differentiation which manifest themselves during maturation: (1) induction of anovulatory sterility due to the loss of the midcycle surge of LH, (2) reduced capacity to show lordotic sexual behavior ("defeminization") and (3) enhancement of male-like mounting behavior ("masculinization"). Prevention of the organizing effects of androgens (orchidectomy, antiandrogens and antiestrogens) produce the opposite of the situation resulting from androgen action: (1) induction of cyclic gonadotropin secretion, (2) induction of lordosis, and (3) failure to mount (McEwen, 1981). Dörner (1976; 1980) and Rohde et al. (1978) have contributed a great deal to this research, using the rat as an experimental model. Dörner has extrapolated these experimental animal observations to the human, equating lordotic behavior in the endocrine-manipulated male rat to homosexual or transsexual behavior in the human male, and mounting behavior in the endocrine-manipulated rat to homosexual or transsexual behavior in the human female. In the case of two males engaged in homosexual acts, both would manifest the human equivalent of lordotic behavior and in the case of two females, both would manifest the equivalent of mounting behavior.

Several conceptual problems of this extrapolation are presented by Meyer-Bahlburg (1982). Neither description is consistent with studies on homosexual behavior (Kinsey Report, 1978). Dörner et al. (1975) proposed that the positive estrogen feedback action on LH secretion in adult life presents a method to evaluate the androgenization of the brain: positive estrogen feedback on LH secretion is present in heterosexual females, absent in heterosexual males, present in homosexual/transsexual males, and attenuated or absent in homosexual and transsexual females. In verifying his working hypothesis gathered from animal research, Dörner described a positive estrogen feedback on LH secretion in transsexual and homosexual men and an attenuation of this in transsexual women.

The latter was supported by work of Seyler et al. (1978).

Dörner et al. (1972; 1976) administered conjugated estrogens (20 mg iv) to 19 homosexual and two transsexual males. LH and FSH levels were measured on five subsequent days. In contrast to hetero- and bisexual men, LH levels in homosexual and transsexual men rose 20–25% above pretreatment values 48–72 hr after estrogen administration. No intra-assay and inter-assay variations in LH measurements, required to judge the significance of an increase as modest as 20–25% above pretreatment values, were reported. Dörner concluded from his data: “sexual deviations in the human may be based, at least in part, on discrepancies between the genetic sex and a sex-specific androgen level during brain differentiation. Therefore a genuine prophylaxis may become possible, if indeed it is desirable at all, in the future by the prevention of such discrepancies during the period of sexual differentiation of the brain.”

Since such far reaching recommendations were made on the basis of Dörner’s animal experimental work and its interpretation and extrapolation to the human situation, a confirmation of this type of investigation in the human is warranted.

In the Netherlands and in our clinic we are able to deal with transsexuality openly. The transsexual subjects were interested in participating in this study, because a biological origin of transsexuality would validate their status and would facilitate their acceptance by society and legal institutions.

To optimally design our protocol on the estrogen positive feedback we first reviewed the published literature to identify the requirements. For women, there are well-defined procedures as to estrogen dosage and duration of exposure (Keye & Jaffe, 1975; Young & Jaffe, 1976). To study this effect in males is more complicated; a great number of studies have been conducted on the role of estrogens in the feedback regulation of gonadotropin secretion in the male. However, they have differed in design, dosage and forms of estrogen used and duration of estrogen administration. Goh et al. (1980), administering  $E_2$  in doses ranging from 0 to 200  $\mu$ g over seven hours, found a suppressive effect on LH secretion in castrated male-to-female transsexuals. In normal men (Kulin & Reiter, 1972; Walsh et al., 1973; Lasley et al., 1976; Dhont et al., 1976; Sawin et al., 1978; Kjeld et al., 1979), administering of ethinylestradiol varying in dosage from 50–100  $\mu$ g/day for five to 10 days found either no effect or a suppressive effect on LH secretion. The LH response to LHRH decreased. Lasley et al. (1976) and D’Agata et al. (1976), administering  $E_2B$  100–400  $\mu$ g/day for three to four days, noted a suppressed LH response to LHRH.

Sherins and Loriaux (1973) and Stewart-Bentley et al. (1974), infusing respectively 90 and 40  $\mu$ g  $E_2$ /day for four days to eugonadal males, observed a suppression of gonadotropins. Neither Van Look et al. (1977) nor Aono et al. (1978) were able to elicit a positive feedback by estrogen administration to subjects with the testicular feminization syndrome. In contrast to these reports, Kulin and Reiter (1976) demonstrated a positive feedback on LH of estrogen administration in four of seven normal adult men and one castrated man. Further, their study produced evidence that estrogen positive feedback on LH is a maturational event occurring during puberty. In a series of well conducted studies Barbarino and de Marinis (1980) and Barbarino et al. (1979; 1982; 1983) reported that an estrogen positive feedback on LH secretion can be evoked in both castrated and intact men provided that blood  $E_2$  levels are comparable to those in women around mid-cycle and that these levels are maintained for 96–120 hr. It also was found that T blunts the magnitude of the LH response to LHRH. These authors concluded that perinatal exposure of the hypothalamic pituitary system to androgens does not completely abolish the capacity of the system to respond to the stimulatory action of estrogens.

Taking the above data into consideration, we took care to administer estrogens in sufficient dosage and duration to test whether a positive feedback on gonadotropins of lesser magnitude could be found in female-to-male transsexuals in comparison to heterosexual female controls, and further to test for positive feedback in male-to-female transsexuals. Since estrogens are known to suppress spermatogenesis and T synthesis, we were not able to recruit either heterosexual or homosexual men to serve as controls for the male-to-female transsexuals.

### Subjects and Methods

The subjects comprised three groups: Group A were six female-to-male transsexuals, aged 18–34 years; group B was six heterosexual female control subjects, aged 23–25 years; and group C was six male-to-female transsexuals, aged 20–36 years.

Groups A and C had applied for gender reassignment treatment. All subjects had been evaluated psychologically for at least one year. Psychological criteria for the diagnosis followed those of the Harry Benjamin International Gender Dysphoria Association. All presented a history of rejection of their originally assigned gender role, with a strong repugnance toward their own genital functioning. Each had an unalterable desire to assume the anatomical characteristics of the other sex in order to adapt their “misshapen” bodies to their self-perceived gender identity. Based on the

data collected during a minimum period of 12 months, all were considered eligible for cross-gender hormone treatment.

Of group A, all subjects had a normal female habitus and genital development. Age of menarche and history of menstrual periods were normal. All of them presented two biphasic basal body temperature charts. Two subjects had been married and had given birth. They both judged their marriages as psychological failures. Presently, like the other four f-to-m transsexuals, they had female partners. When questioned, the partners labelled themselves as heterosexual, and they viewed their transsexual partners as boyfriends and potential husbands. On the first examination, between days 5 and 10 of their cycles, normal values of LH, FSH, prolactin, T and E<sub>2</sub> had been found. Each had a 46 XX karyotype.

Of the six m-to-f transsexuals (group C), one had been married and had fathered. His wife was prepared to continue their relationship. Another m-to-f transsexual had a female companion, and their relationship was considered lesbian. The other four m-to-f transsexuals had some experience in a male homosexual milieu and had concluded that they definitely were not male homosexuals. Homosexual activities in which they were perceived as men clearly had not been satisfactory. They now had heterosexual male partners. These six m-to-f transsexuals had a normal male habitus, including male sexual hair distribution and normal genital development. All regularly had erections and ejaculations. In each a 46 XY karyotype was found. On the first examination, normal values of LH, FSH, prolactin, T and E<sub>2</sub> were found.

The group of female controls was composed of fourth and fifth year medical students. Each considered herself exclusively heterosexual. All had a normal age of menarche and a regular menstrual cycle. Each presented one biphasic basal body temperature chart. None had been taking any sex steroid for at least one year prior to our investigation.

An identical test procedure, performed on five consecutive days, was employed in all subjects. In the f-to-m transsexuals and the female controls, testing was commenced on day 5 of their menstrual cycle (day 1 of the menstrual bleeding being the first day of the cycle).

From day 1 through day 5, blood for determination of E<sub>2</sub>, T, LH and FSH was collected between 0800 and 0900 hr and between 1700 and 1800 hr. Sampling was immediately followed by administration of E<sub>2</sub>B (Dimenformon, Organon). As to the dose of E<sub>2</sub>B, it was decided to follow the results of Keye and Jaffe (1975) and Young and Jaffe (1976). They established that an E<sub>2</sub>B dose of 3.75–5.00 µg/kg/12 hr for a duration of 84 to 132 hr was very likely to elicit an

increased response of gonadotropins to LHRH in women. We administered 4.5 µg/kg/12 hr.

On day 1 of the test, preceding estrogen administration, and on day 5 following the course of estrogen administration, all subjects underwent a LHRH-stimulation test. Blood for determination of LH and FSH was sampled at 0, 30 and 60 min in connection with the administration of LHRH (Ayerst) (100 µg) as a bolus.

LH, FSH, T and E<sub>2</sub> were determined by radioimmunoassay. Student's t-tests for paired and unpaired observations were used for statistical analysis.

The aim and the nature of the experiment was explained to all subjects. In our clinic, transsexual subjects are guaranteed treatment regardless of participation in studies. This is with the intent of recruiting subjects free of hormone use. To all participants it was obvious that any previous hormone use could invalidate the results. The study was approved by the hospital ethics committee.

## Results

T levels did not differ between f-to-m transsexuals ( $1.6 \pm 0.3$  nmol/l) and female controls ( $1.7 \pm 0.4$  nmol/l). Neither did basal LH or FSH levels at the beginning of the test period: LH ( $5.3 \pm 1.3$  U/l) and FSH ( $4.6 \pm 1.2$  U/l) in f-to-m transsexuals and LH ( $5.0 \pm 1.6$  U/l) and FSH ( $4.0 \pm 1.6$  U/l) in controls. Estradiol levels also were similar in f-to-m transsexuals ( $0.10 \pm 0.02$  nmol/l) and controls ( $0.09 \pm 0.02$  nmol/l).

Results of the gonadotropin response to estrogen administration in the f-to-m transsexuals (Group A) and the female controls (Group B) are presented for each subject individually. The values show an oscillating nature, so that pooling and calculating mean values of the data could obscure the responses. A positive estrogen feedback was defined as a 100% increase of LH levels above the value measured at the beginning of the test. In all m-to-f transsexuals and in all female controls, LH (and sometimes FSH) showed an increase from 36 to 48 hr onwards through the five day course of estrogen administration (figures 84.1 and 84.2). The response of LH, expressed as area under the curve, proved to be greater in f-to-m transsexuals ( $50.9 \pm 7.0$  U/l/day) than in controls ( $35.6 \pm 6.0$  U/l/day) ( $p < 0.01$ ).

Plasma estradiol levels in the three groups are presented in figure 84.3.

The responses of LH and FSH to LHRH did not differ significantly between groups A and B (figures 84.4 and 84.5). After estrogen administration the increase of the LH response to LHRH compared to baseline in the f-to-m transsexuals was from  $390 \pm 60$  to  $510 \pm 64$  U/l/min ( $p < 0.05$ ) and in the controls

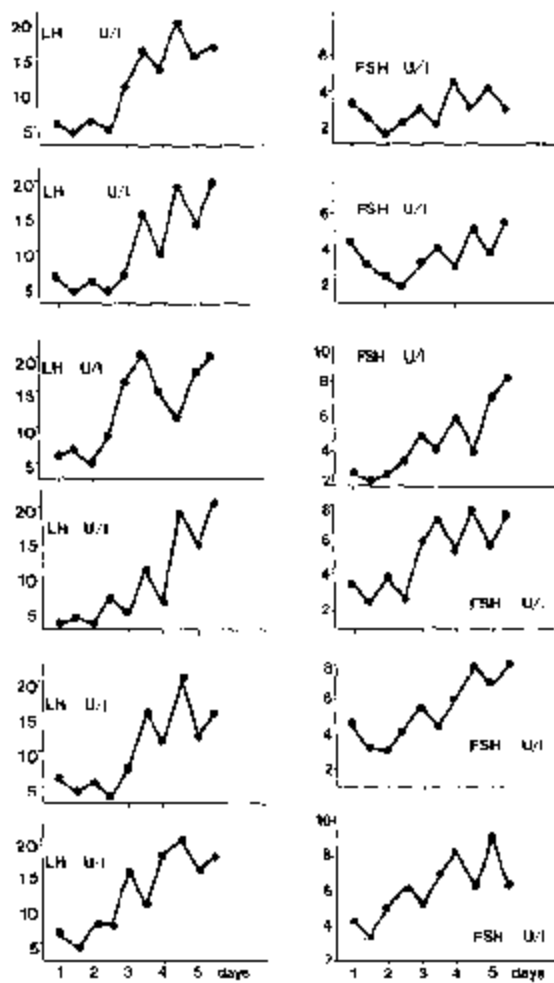


Figure 84.1

Effect of administration of E<sub>2</sub>B (4.5 µg/kg) two times daily on plasma LH and FSH levels in six f-to-m transsexuals.

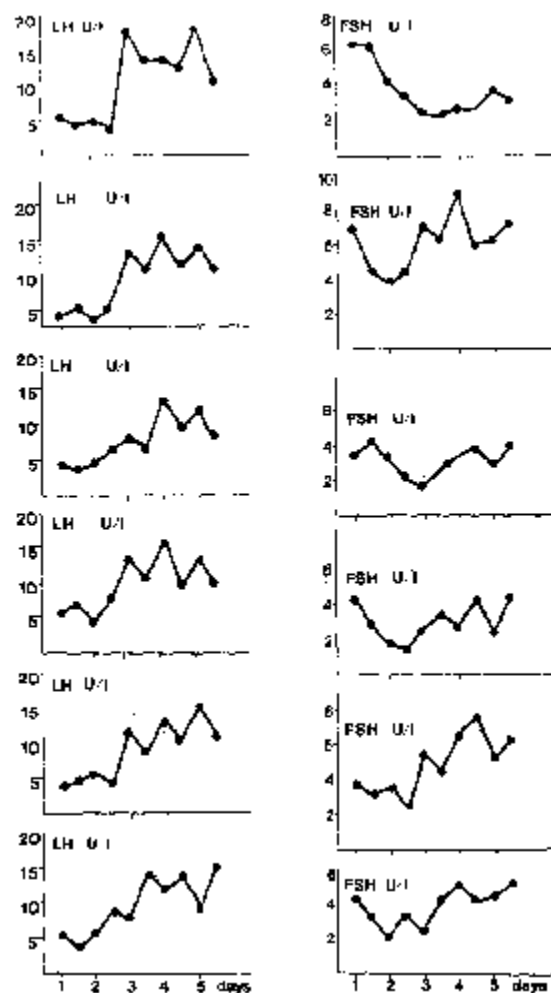


Figure 84.2

Effect of administration of E<sub>2</sub>B (4.5 µg/kg) two times daily on plasma LH and FSH levels in six heterosexual women.

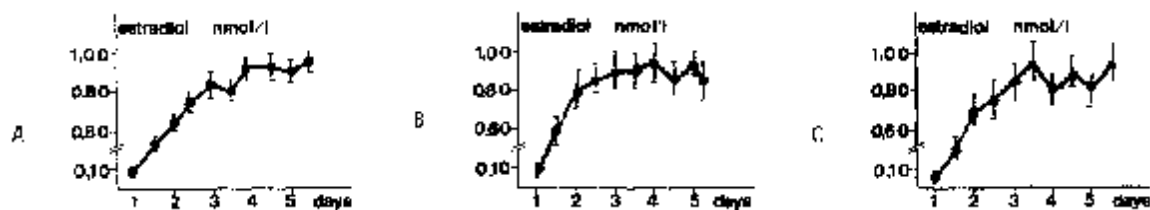
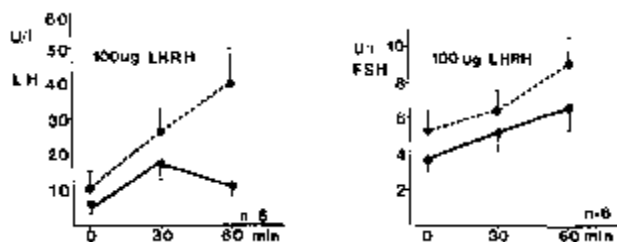
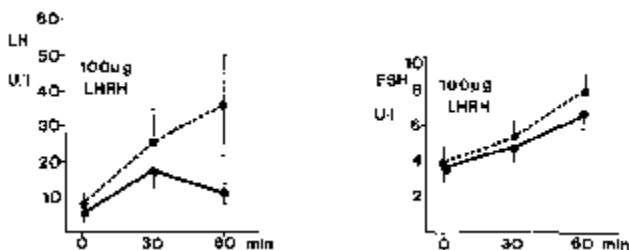


Figure 84.3

Plasma estradiol levels (mean values  $\pm$  S.E.M.) in (A) six f-to-m transsexuals, (B) six heterosexual women and (C) six m-to-f transsexuals, upon administration of E<sub>2</sub>B (4.5 µg/kg) two times daily.



**Figure 84.4**  
LH and FSH responses (mean values  $\pm$  S.E.M.) to administration of LHRH (100 µg) in six f-to-m transsexuals before (—) and after (---) administration of E<sub>2</sub>B (4.5 µg/kg) two times daily for five days.



**Figure 84.5**  
LH and FSH responses (mean values  $\pm$  S.E.M.) to administration of LHRH (100 µg) in six heterosexual women before (—) and after (---) administration of E<sub>2</sub>B (4.5 µg/kg) two times daily for five days.

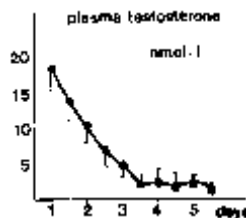
was from  $350 \pm 70$  to  $490 \pm 69$  U/l/min ( $p < 0.05$ ). A further point of interest was the change in characteristics of the LH response to LHRH. Whereas LH levels were maximal 30 min after LHRH before E<sub>2</sub>B administration, the value at 60 min after LHRH was the peak after E<sub>2</sub>B. (It is possible that higher levels could have been recorded later than 60 min after LHRH administration.)

In the m-to-f transsexuals, T ( $18.0 \pm 3.9$  nmol/l), LH ( $5.1 \pm 1.2$  U/l) and FSH ( $6.0 \pm 1.1$  U/l) levels were comparable to those found in eugonadal men in our laboratory. Upon administration of E<sub>2</sub>B, an immediate fall in T levels occurred (figure 84.6). From 36 hours onward a significant fall in LH levels occurred, whereas a significant decrement of FSH began 12 hr after E<sub>2</sub>B administration (figure 84.7).

The responses of LH and FSH to LHRH decreased significantly after estrogen administration: LH  $306 \pm 50$  U/l/min to  $90 \pm 30$  U/l/min ( $p < 0.01$ ) and FSH  $120 \pm 29$  U/l/min to  $30 \pm 9$  U/l/min ( $p < 0.05$ ) (figure 84.8).

## Discussion

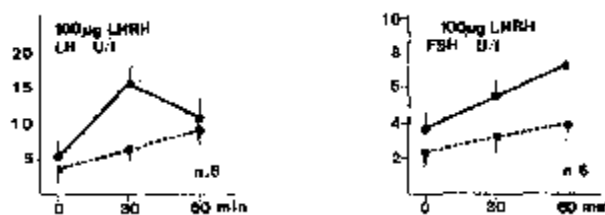
The literature survey in this area strongly indicated that the dose and duration of exposure to estrogens play a critical role in the feedback control mechanism



**Figure 84.6**  
Plasma T levels (mean values  $\pm$  S.E.M.) in six f-to-m transsexuals upon administration of E<sub>2</sub>B (4.5 µg/kg) two times daily.



**Figure 84.7**  
Plasma LH and FSH levels (mean values  $\pm$  S.E.M.) in six m-to-f transsexuals upon administration of E<sub>2</sub>B (4.5 µg/kg) two times daily.



**Figure 84.8**  
LH and FSH responses (mean values  $\pm$  S.E.M.) to administration of LHRH (100 µg) in six m-to-f transsexuals before (—) and after (---) administration of E<sub>2</sub>B (4.5 µg/kg) two times daily for five days.

regulating pituitary gonadotropin secretion. In our present study we elected to administer E<sub>2</sub>B (4.5 µg/kg) every 12 hours for five days. This dose and duration appeared to be sufficient to evoke the expected response in women.

Several interesting points can be made from our investigations. Estrogen administration increased LH secretion and evoked an unequivocally positive feedback in a group of f-to-m transsexuals. Although this pattern of response also was observed in a group of heterosexual female controls, the magnitude of the LH response was significantly higher in the f-to-m transsexuals than in the controls. In no case was there a negative feedback or a positive feedback of a lesser magnitude in the f-to-m transsexuals. We have no definitive explanation to offer for the observation that in the f-to-m transsexuals the positive estrogen feedback effect was greater than in the female controls. The only possible difference is in their ages (four f-to-m transsexuals were between 28 and 36 years of age,

whereas the controls were 23 to 25 years old). Another factor which might have contributed to these differences is the use of oral contraceptive hormones. Three of the controls had used oral contraceptives 15 months prior to entering our protocol. However, we do not feel that this may have played a role, since the controls had returned to regular menstrual cycles, with biphasic basal body temperatures. The T levels in the f-to-m transsexuals and in the heterosexual female controls did not differ significantly. This is contrary to some data that f-to-m transsexuals have higher T levels than heterosexual female controls. Additionally the LH responses to LHRH administration in both groups, unlike the estrogen positive feedback responses, were similar. Taken together, the data indicate that the f-to-m group could not be distinguished from the heterosexual females on the basis of blood hormone levels and their responses to standard hormone stimulation tests.

In this respect our observations differ from those of Seyler et al. (1978). The reason for this, other than their use of a different estrogenic compound (DES), is not apparent. A recent investigation in f-to-m transsexuals, also employing DES, could not differentiate the transsexuals from a control woman, which is in agreement with our finding (Wiesen & Futterweit, 1983).

Striking decreases in T, LH, and FSH levels were observed in the m-to-f transsexuals when estrogen was administered. There also was a substantial decrease in LH and FSH levels after LHRH administration following estrogen administration, in contrast to those observed in the f-to-m transsexuals and the female controls. Thus, there was neither an increase in LH levels nor an increment in LH levels after LHRH in the estrogen-primed m-to-f transsexuals. This observation does not support the hypothesis that m-to-f transsexuals can be distinguished by their responses to hormonal manipulations, specifically by a positive estrogen feedback on gonadotropins.

It is evident that the present observations differ from those of Dörner (1976; 1980) and Dörner et al. (1976) in several respects. We elected to administer estrogens in a continuous fashion, considering estrogen positive feedback as an increase in LH levels in the presence of high circulating levels of estrogen for a sufficient duration. In the absence of any information on estrogen blood levels, it is difficult to accept the positive estrogen feedback on LH levels observed by Dörner (1976) and Dörner et al. (1976) following a single, though high dosage, estrogen administration intravenously in m-to-f transsexuals. Unfortunately, Dörner did not consider the possibility of a rebound phenomenon observed by several others following discontinuation of estrogen administration (Stewart-Bentley et al., 1974; Sherins & Loriaux, 1973; Kjeld et al., 1979). Additionally, it could be noted from Dörner's data that

the magnitude of the LH increase was similar to the increase in LH levels due to a rebound phenomenon observed by others. A further point that could be made regarding the extrapolation of Dörner's observations in the rat to the human is the study of Karsch et al. (1973), who demonstrated that in primates (rhesus monkeys), in contrast to rodents, exposure of the hypothalamo-hypophyseal unit to androgens throughout fetal and postnatal development does not prevent the differentiation of the control system that governs cyclic gonadotropin secretion.

The results reported by Seyler et al. (1978) in heterosexual males agree with our observations in m-to-f transsexuals: no sign of a positive feedback upon estrogen administration could be found in either group. While their study agrees with ours, both Kulin and Reiter (1976) and Barbarino et al. (1983) reported a positive estrogen feedback in men, employing study designs and estrogen doses similar to ours. E<sub>2</sub> levels in the study of Kulin and Reiter were not different from those in our study, whereas those in the Barbarino et al. study were higher.

The greater duration of E<sub>2</sub> administration and follow up of LH levels by Kulin and Reiter (1976) could possibly explain why they found a positive estrogen feedback. Our five-day period of E<sub>2</sub> administration might prove to be insufficient to activate the hypothalamic-pituitary unit and induce an LH release. Rather, a longer period of observation and/or higher doses of E<sub>2</sub> might be necessary to either prove or disprove the presence of a positive estrogen feedback in the m-to-f transsexual subjects. The fact that the LH response to LHRH after five days of E<sub>2</sub> administration in the m-to-f transsexuals was decreased does not necessarily contradict this, since in women also, after E<sub>2</sub> administration, LH levels decline initially, reverting eventually to a positive feedback reaction.

We plan to determine whether an estrogen positive feedback response can be observed in long-term orchidectomized m-to-f transsexuals, in whom T levels are expected to be minimal. Whereas we could not establish the existence of an estrogen positive feedback in our group of m-to-f transsexuals, it could be so, in view of the studies of Karsch et al. (1973), Kulin and Reiter (1976) and Barbarino et al. (1983), that the presence of this phenomenon in the male is not a distinctive feature of the m-to-f transsexual.

We do not interpret our findings to the extent of dismissing the possibility of an endocrine basis of transsexuality, but transsexuality is apparently not distinguished by the action of estrogen on the hypothalamo-pituitary-gonadal axis.

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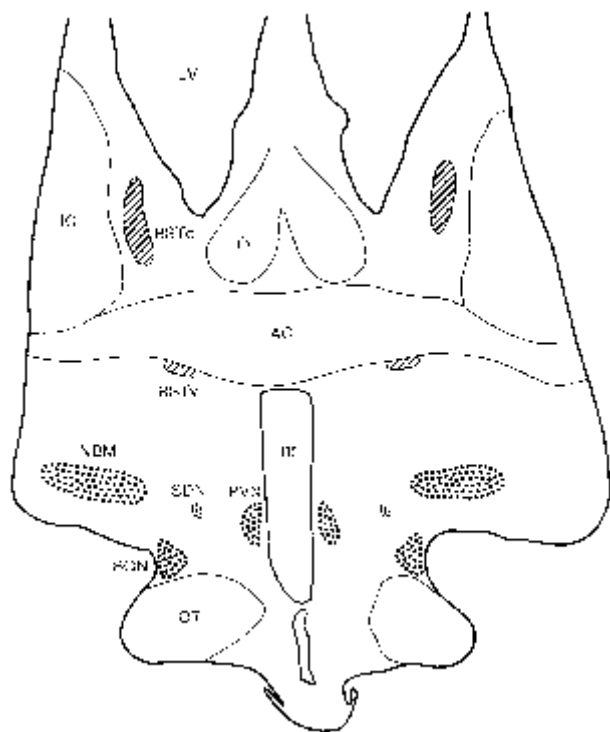
Transsexuals have the strong feeling, often from childhood onwards, of having been born the wrong sex. The possible psychogenic or biological aetiology of transsexuality has been the subject of debate for many years (1, 2). Here we show that the volume of the central subdivision of the bed nucleus of the stria terminalis (BSTc), a brain area that is essential for sexual behaviour (3, 4), is larger in men than in women. A female-sized BSTc was found in male-to-female transsexuals. The size of the BSTc was not influenced by sex hormones in adulthood and was independent of sexual orientation. Our study is the first to show a female brain structure in genetically male transsexuals and supports the hypothesis that gender identity develops as a result of an interaction between the developing brain and sex hormones (5, 6).

Investigation of the genetics, gonads, genitalia or hormone level of transsexuals has not, so far, produced any results that explain their status (1, 2). In experimental animals, however, the same gonadal hormones that prenatally determine the morphology of the genitalia also influence the morphology and function of the brain in a sexually dimorphic fashion (6, 7). This led to the hypothesis that sexual differentiation of the brain in transsexuals might not have followed the line of sexual differentiation of the body as a whole. In the past few years, several anatomical differences in relation to sex and sexual orientation have been observed in the human hypothalamus (see ref. 6 for a review), but so far no neuroanatomical investigations have been made in relation to the expression of cross-gender identity (transsexuality).

We have studied the hypothalamus of six male-to-female transsexuals (T1–T6); this material was collected over the past eleven years. We searched for a brain structure that was sexually dimorphic but that was not influenced by sexual orientation, as male-to-female transsexuals may be “oriented” to either sex with respect to sexual behaviour. Our earlier observations showed that the paraventricular nucleus (PVN), sexually dimorphic nucleus (SDN) and suprachiasmatic nucleus (SCN) did not meet these criteria (ref. 6 and unpublished data). Although there is no accepted

animal model for gender-identity alteration, the bed nucleus of the stria terminalis (BST) turned out to be an appropriate candidate to study for the following reasons. First, it is known that the BST plays an essential part in rodent sexual behaviour (3, 4). Not only have oestrogen and androgen receptors been found in the BST (8, 9), it is also a major aromatization centre in the developing rat brain (10). The BST in the rat receives projections mainly from the amygdala and provides a strong input in the preoptic–hypothalamic region (11, 12). Reciprocal connections between hypothalamus, BST and amygdala are also well documented in experimental animals (13–15). In addition, sex differences in the size and cell number of the BST have been described in rodents which are influenced by gonadal steroids in development (16–18). Also, in humans a particular caudal part of the BST (BNST-dspm) has been reported to be 2.5 times larger in men than in women (19).

The localization of the BST is shown in figure 85.1. The central part of the BST (BSTc) is characterized by its somatostatin cells and vasoactive intestinal polypeptide (VIP) innervation (20). We measured the volume of the BSTc on the basis of its VIP innervation (figure 85.2). The BSTc volume in heterosexual men ( $2.49 \pm 0.16 \text{ mm}^3$ ) was 44% larger than in heterosexual women ( $1.73 \pm 0.13 \text{ mm}^3$ ) ( $P < 0.005$ ) (figure 85.3). The volume of the BSTc of heterosexual and homosexual men did not differ in any statistically significant way ( $2.81 \pm 0.20 \text{ mm}^3$ ) ( $P = 0.26$ ). The BSTc was 62% larger in homosexual men than in heterosexual women ( $P < 0.005$ ). AIDS did not seem to influence the size of the BSTc: the BSTc size of two heterosexual AIDS-infected women and three heterosexual AIDS-infected men remained well within the range of the corresponding reference group (figure 85.3). The AIDS-infected heterosexuals were therefore included in the corresponding reference group for statistical purposes. A small volume of the BSTc ( $1.30 \pm 0.23 \text{ mm}^3$ ) was found in the male-to-female transsexuals (figure 85.3). Its size was only 52% of that found in the reference males ( $P < 0.005$ ) and 46% of the BSTc of homosexual males ( $P < 0.005$ ). Although the mean BSTc



**Figure 85.1**

Schematic frontal section through two subdivisions of the bed nucleus of the stria terminalis (BST). III, third ventricle; AC, anterior commissure; BSTc and BSTv, central and ventral subdivisions of the BST; FX, fornix; IC, internal capsule; LV, lateral ventricle; NBM, nucleus basalis of Meynert; OT, optic tract, PVN, paraventricular nucleus; SDN, sexually dimorphic nucleus; SON, supraoptic nucleus.

volume in the transsexuals was even smaller than that in the female group, the difference did not reach statistical significance ( $P = 0.13$ ). The volume of the BSTc was not related to age in any of the reference groups studied ( $P > 0.15$ ), indicating that the observed small size of the BSTc in transsexuals was not due to the fact that they were, on average, 10 to 13 years older than the hetero- and homosexual men.

The BST plays an essential role in masculine sexual behaviour and in the regulation of gonadotrophin release, as shown by studies in the rat (3, 4, 21). There has been no direct evidence that the BST has such a role in human sexual behaviour, but our demonstration of a sexually dimorphic pattern in the size of the human BSTc, which is in agreement with the previously described sex difference in a more caudal part of the human BST (BNST-dspm) (19), indicates that this nucleus may also be involved in human sexual or reproductive functions. It has been proposed that neurochemical sex differences in the rat BST may be due to effects of sex hormones on the brain during development and in adulthood (22, 23). Our data from humans however, indicate that BSTc volume is not affected by varying sex hormone levels in adulthood. The BSTc

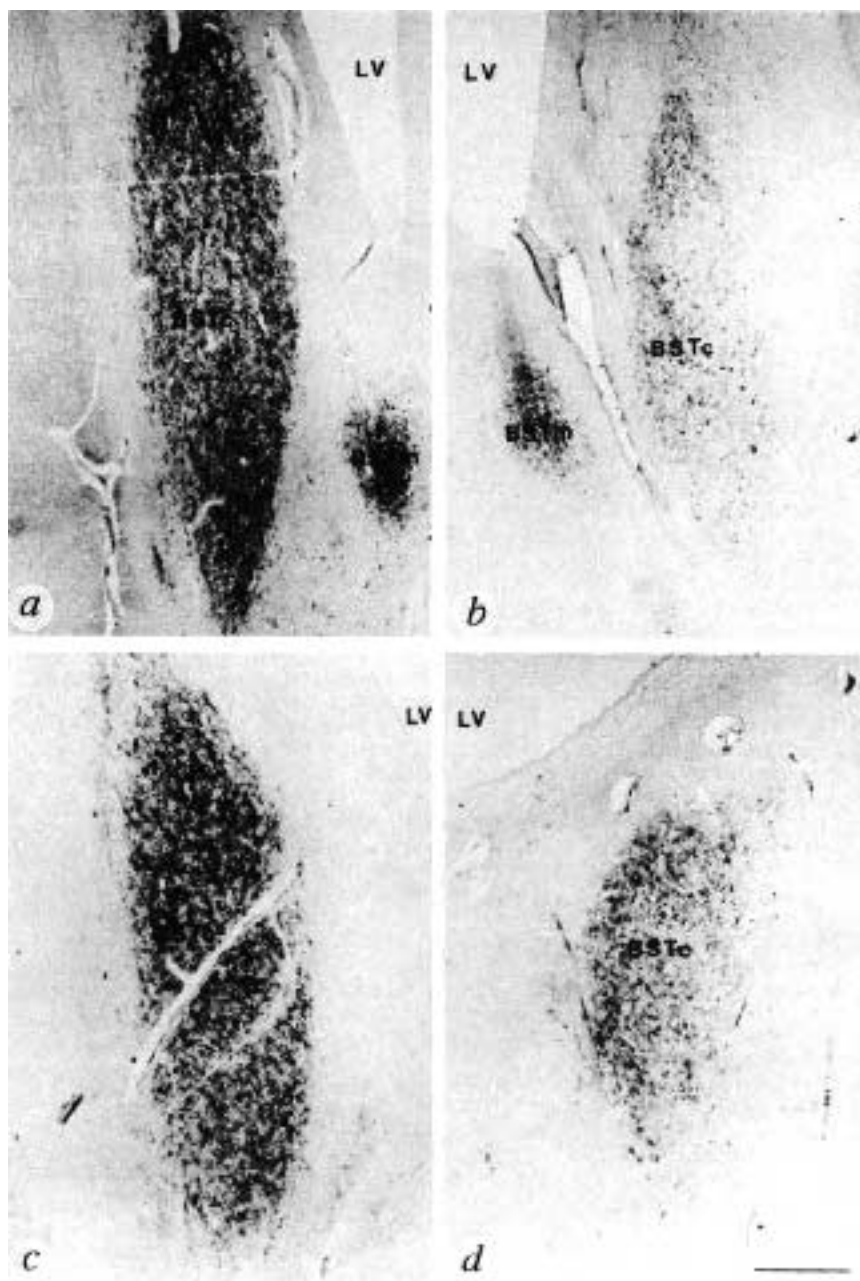
volume of a 46-year-old woman, who had suffered for at least one year from a tumour of the adrenal cortex that produced very high blood levels of androstenedione and testosterone, was within the range of that of other women (figure 85.3; S1). Furthermore, two postmenopausal women (aged over 70 years) showed a completely normal female-sized BSTc (figure 85.3; M1, M2). As all the transsexuals had been treated with oestrogens, the reduced size of the BSTc could possibly have been due to the presence of high levels of oestrogen in the blood. Evidence against this comes from the fact that transsexuals T2 and T3 both showed a small, female-like BSTc (figure 85.3), although T2 stopped taking oestrogen about 15 months before death because her prolactin levels were too high and T3 stopped hormone treatment as a sarcoma was found about 3 months before death; also a 31-year-old man who suffered from a feminizing adrenal tumour which induced high blood levels of oestrogen, nevertheless had a very large BSTc (figure 85.3; S2).

Our results might also be explained if the female-sized BSTc in the transsexual group was due to a lack of androgens, because they had all been orchidectomized except for T4. We therefore studied two other men who had been orchidectomized because of cancer of the prostate (one and three months before death: S4 and S3, respectively), and found that their BSTc sizes were at the high end of the normal male range. The BSTc size of the single transsexual who had not been orchidectomized (T4) ranged in the middle of the transsexual scores (figure 85.3). Not only were five of the transsexuals orchidectomized, they all used the antiandrogen compound cyproterone acetate (CPA). A CPA effect on the BSTc does not seem likely, because T6 had not taken CPA for the past 10 years, and T3 took no CPA during the 2 years before death and still had a female-sized BSTc.

In summary, our observations suggest that the small size of the BSTc in male-to-female transsexuals cannot be explained by differences in adult sex hormone levels, but is established during development by an organizing action of sex hormones, an idea supported by the fact that neonatal gonadectomy of male rats and androgenization of female rats induces significant changes in the number of neurons of the BST and suppresses its sexual dimorphism (17, 18).

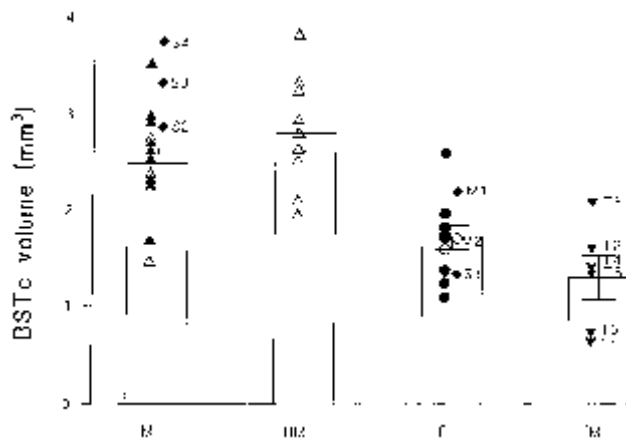
Considered together with information from animals, then our study supports the hypothesis that gender identity alterations may develop as a result of an altered interaction between the development of the brain and sex hormones (5, 6). The direct action of genetic factors should also be considered on the basis of animal experiments (24).

We found no relationship between BSTc size and the sexual orientation of transsexuals, that is, whether they



**Figure 85.2**

Representative sections of the BSTc innervated by vasoactive intestinal polypeptide (VIP). *a*, Heterosexual man; *b*, heterosexual woman; *c*, homosexual man; *d*, male-to-female transsexual. Scale bar, 0.5 mm. LV, lateral ventricle. Note there are two parts of the BST in *a* and *b*: small medial subdivision (BSTm) and large oval-sized central subdivision (BSTc).



**Figure 85.3**

Volume of the BSTc innervated by VIP fibres in presumed heterosexual males (M), homosexual males (HM), presumed heterosexual females (F) and male-to-female transsexuals (TM). The six transsexuals are numbered T1–T6. The patients with abnormal sex hormone levels are numbered S1–S4. M1 and M2, postmenopausal women. Bars indicate mean  $\pm$  s.e.m. Open symbols: individuals who died of AIDS.

**METHODS.** Brains of 42 subjects matched for age, postmortem time and duration of formalin fixation were investigated. The autopsy was performed after obtaining permission. For immunocytochemical staining of VIP, paraffin sections were hydrated and rinsed in Tris-buffered-saline TBS: 0.05 M Tris, 0.9% NaCl, pH 7.6). Sections were incubated with 200  $\mu$ l anti-VIP 1:1,000 in 0.5% Triton in TBS overnight at 4 °C. Immunocytochemical and morphometric procedures have been described (25–27). In brief, serial 6  $\mu$ m sections of the BSTc were studied by means of a digitizer (Calcomp 2000) connected to a HP-UX 9.0, using a Zeiss microscope equipped with a 2.5 $\times$  objective and with 10 $\times$  (PLAN) oculars. Staining was performed on every fiftieth section with anti-VIP. The rostral and caudal borders of the BSTc were assessed by staining every tenth section in the area. The volume of the BSTc was determined by integrating all the area measurements of the BSTc sections that were innervated by VIP fibres. In a pilot study, the size of the BSTc was measured on both sides in 8 subjects (5 females and 3 males) and no left–right asymmetries were observed: the left BSTc ( $1.71 \pm 0.16$  mm<sup>3</sup>) was comparable in size to that of the right BSTc ( $1.83 \pm 0.30$  mm<sup>3</sup>) ( $P = 0.79$ ). No asymmetry was observed in the BNST-dspm either (19). The rest of our study was therefore performed on one side of the brain only. Brain weight of the male transsexuals ( $1,385 \pm 78$  g) was not different from that of the reference males ( $1,453 \pm 25$  g) ( $P = 0.61$ ) or that of the females ( $1,256 \pm 35$  g) ( $P = 0.23$ ). The causes of death of the transsexuals were suicide (T1), cardiovascular disease (T2, T6), sarcoma (T3), AIDS, pneumonia, pericarditis (T4) and hepatic failure (T5). Sexual orientation of the subjects of the reference group (12 men and 11 women) was generally not known, but most of them were presumed heterosexual. Sexual orientation of 9 homosexuals was registered in the clinical records (28). Differences among the groups were tested two-tailed using the Mann–Whitney U-test. A 5% level of significance was used in all statistical tests.

were male-oriented (T1, T6), female-oriented (T3, T2, T5) or both (T4). Furthermore, the size of the BSTc of heterosexual men and homosexual men did not differ, which reinforced the idea that the reduced BSTc size is independent of sexual orientation. In addition, there was no difference in BSTc size between early-onset (T2, T5, T6) and late-onset transsexuals (T1, T3), indicating that the decreased size is related to the gender identity alteration per se rather than to the age at which it becomes apparent. Interestingly, the very small BSTc in transsexuals appears to be a very local brain difference. We failed to observe similar changes in three other hypothalamic nuclei, namely PVN, SDN or SCN in the same individuals (unpublished data). This might be due to the fact that these nuclei do not all develop at the same time, or to a difference between these nuclei and the BST with respect to the presence of sex hormone receptors or aromatase. We are now studying the distribution of sex hormone receptors and the aromatase activity in various hypothalamic nuclei in relation to sexual orientation and gender.

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Animal experiments and observations in human brains have convincingly shown that sexual differentiation not only concerns the genitalia but also the brain (1, 2). The strongly connected and sexually differentiated hypothalamus, septum, bed nucleus of the stria terminalis (BST), and amygdala are implicated in sexually dimorphic patterns of reproductive and nonreproductive behaviors (2–18).

Gender identity (i.e. the feeling to be male or to be female) is an important trait of a subject. Transsexuals experience themselves as being of the opposite sex, despite having the biological characteristics of one sex (19–21). In line with the hypothesis that in transsexuals sexual differentiation of the brain contrasts with that of the genetic and physical characteristics of sex, our group has recently found that the size of the central subdivision of the BST (BSTc) was within the female range in genetically male-to-female transsexuals (22). In that study the, BSTc was defined on the basis of its vasoactive intestinal polypeptide innervation, which is probably mainly derived from the amygdala (23). A crucial question resulting from that study was, therefore, whether the difference according to gender in the BSTc is based on a neuronal difference in the BSTc itself or rather a reflection of a difference in innervation from the amygdala. To see whether the BSTc itself has a neuronal organization that is opposite to that of the genetic and genital characteristics of transsexuals, we determined the number of somatostatin (SOM)-expressing neurons in the BSTc, which is the major neuronal population in this structure (23).

## Materials and Methods

### Patients

In the present study, 42 brains of patients were analyzed (for an overview see table 86.1). The brains of 34 reference subjects (9 presumed heterosexual males, 9 homosexual males, 10 presumed heterosexual females, and 6 male-to-female transsexuals) ranging from 20–53 yr of age, together with six brains (three males and three females) of patients with sex hormone disorders were obtained at autopsy, after the required permis-

sions had been obtained. Twenty-six of the reference subjects were the same as used in the earlier study of Zhou et al. (22), whereas eight new patients (five females, two males, and one homosexual man) were included because not enough sections were left for the present study. A Turner syndrome patient (S6) and a castrated (orchietomized) male patient (S5) were included in the sex hormone disorder group [ $n = 6$ ; see the legend to figure 86.1; S1, S2, S3, and M2 were also used in the study of Zhou et al. (22)]. A nontreated individual with strong cross-gender identity feelings (S7), which were already present since his earliest childhood, was also analyzed. In addition, we had the exceptional opportunity to be able to study the first collected brain ever of a female-to-male transsexual (FMT). The brains were matched for age, postmortem time, and duration of formalin fixation. Neuropathology of all subjects was systematically performed by Dr. W. Kamphorst (Free University, Amsterdam, The Netherlands), Dr. D. Troost (Academic Medical Centre of the University of Amsterdam, Amsterdam, The Netherlands), or Prof. F. C. Stam (Netherlands Brain Bank, Amsterdam, The Netherlands). Subjects had no primary neurological or psychiatric diseases, unless stated otherwise.

### Histology

Brains were weighed, generally followed by 37 days of fixation in 4% formaldehyde at room temperature. The hypothalamic area was subsequently dissected, dehydrated, and embedded in paraffin. Serial 6- $\mu$ m frontal sections were cut on a Leitz microtome, mounted on SuperFrost/Plus (Menzel-Gläser, Braunschweig, Germany; Art. No. 041300) slides, and subsequently dried overnight on a hot plate at 58 C.

### Immunocytochemistry

Sections were hydrated and rinsed in aquadest  $2 \times 5$  min and Tris-buffered saline [TBS; 0.05 M Tris, and 0.9% NaCl (pH 7.6)] for 30 min. To enhance antigen retrieval [for a review see Shi et al. (24)], sections were put in a plastic jar [filled with a Citrate 0.05 M (pH 4.0) buffer solution] and heated to boiling (120 C) for 10

**Table 86.1**  
Brain material

NBB Patient Number	Age (yr)	Brain Weight (g)	Postmortem Delay (h)	Fixation Time (Days)	Clinicopathological Diagnosis
<i>Reference men (n = 9)</i>					
86042	28	1450	24	46	Guillain-Barré syndrome
84015	29	1400	13	41	Congenital heart disease; cardiac failure
94040	20	1490	8	82	B-cell lymphoma; viral pneumonia, hemorrhage, heart failure
89042	30	1340	30	26	AIDS; disseminated non-Hodgkin's lymphoma
84023	37	1370	39	35	Bronchopneumonia
88011	41	1500	21	33	Suicide
92011	47	1520	<89	77	Pneumococcal sepsis
95102	53	1383	10	33	Aorta dissection
86048	30	1430	8	35	AIDS, pneumocystic carinii pneumonia, lung tuberculosis, toxoplasmosis, heroin addiction
<i>Reference women (n = 10)</i>					
85027	29	1150	13	60	Corrected Fallots' teratology; cardiac failure, hepatic coma
85041	28	ND	5	44	Cardiogenic shock
84025	23	1300	<10	35	Acute myeloid leukemia
86032	33	1035	<41	20	Adenocarcinoma with metastasis
92037	32	1280	30	45	Bronchopneumonia
88096	34	1400	<12	31	AIDS; disseminated histoplasmosis
84002	36	1420	86	51	Multiple fractures; rupture of thoracic aorta
80002	46	1300	3	ND	Ovarium carcinoma
89104	49	1260	<41	32	Septic shock; lung carcinoma
86039	53	1410	34	17	Myelocytic leukemia; blastomatosis
<i>Homosexuals (n = 9)</i>					
89031	25	1530	23	28	AIDS; pneumonia
88009	30	1480	5	27	AIDS; cytomegalic infections
87015	30	1640	24	26	AIDS; pneumocystic carinii pneumonia
87080	39	1320	24	28	AIDS; progressive multifocal leukoencephalopathy
88121	42	1340	19	30	AIDS; cytomegalic meningoencephalitis
86023	43	1260	2	100	AIDS; disseminated Kaposi's sarcoma and pneumonia
88087	41	1240	12	34	AIDS; bronchopneumonia, cytomegalic infections and toxoplasmosis
86046	32	1440	49	11	AIDS; pneumocystic carinii pneumonia
89024	21	1430	<49	25	AIDS; mycobacterial infections, pneumonia, cerebrovascular accident
<i>Male-to-female transsexuals (n = 6)</i>					
84020 (T1)	50	1380	ND	30	Suicide
84037 (T2)	44	1450	ND	34	Cardiovascular death
88064 (T3)	43	1540	ND	ND	Sarcoma
93042 (T4)	36	1145	21	31	AIDS, pneumonia, pericarditis, cytomegaly in brain
93070 (T5)	53	1500	96	34	Acute fatty liver due to alcohol
95018 (T6)	48	1198	24	36	Cardiovascular death, cardiac arrest
<i>Sex hormone disorder cases (n = 6)</i>					
83004 ♀ (S1)	46	1260	ND	34	Adrenocortical carcinoma; postoperative hemorrhage
89103 ♂ (S3)	67	1290	ND	28	Pancreaticocarcinoma; prostate carcinoma; orchidectomy
91044 ♀ (S6)	25	1200	13	103	Turner syndrome (XO); related cardiovascular problems; decompensatio cordis
94090 ♂ (S5)	86	1663	3	93	Lung and prostate carcinoma; orchidectomy; septic shock
89077 ♀ (M2)	73	1090	<48	33	AIDS; pneumonia; epilepsy
91005 ♂ (S2)	31	1377	34	35	Feminizing adrenocortex carcinoma
<i>Nontreated male with cross-gender identity feelings (n = 1)</i>					
96088 ♂ (S7)	84	1433	41	38	Lung carcinoma
<i>FMT (n = 1)</i>					
98138	51		4	32	Cachexia

ND, Not determined.

min at 700 W in a microwave oven (Miele Electronic M696, Darmstadt, Germany). After cooling down for about 10 min, the sections were washed in TBS for  $3 \times 10$  min and preincubated in TBS (pH 7.6) containing 5% nonfat dry milk (Elk, Campina bv., Eindhoven, The Netherlands) to reduce background staining. Subsequently, a circle was drawn around the sections with a Dakopen (Glostrup, Denmark; Code No. S 2002) to prevent the antibody from diffusing. The sections were: 1) incubated with 300- $\mu$ L rabbit antisomatostatin [SOMAAR, 8/2/89; dilution 1:500; for details and specificity see Van de Nes et al. (25)] in 0.5% Triton X-100 (Sigma, Steinheim, Germany), 0.25% gelatin, and 5% nonfat dry milk TBS solution [supermix-milk (pH 7.6)] overnight at 4 C; 2) washed in TBS-milk  $3 \times 10$  min, followed by a second incubation with goat antirabbit IgG antiserum (Betsie, NIBR, Amsterdam, The Netherlands; dilution 1:100) in supermix for 60 min; 3) washed in TBS-milk  $3 \times 10$  min; 4) incubated with rabbit peroxidase-antiperoxidase (dilution 1:1000 in supermix) for 30 min; 5) rinsed  $3 \times 10$  min in 0.05 M Tris-HCL (Merck, Darmstadt, Germany; pH 7.6); 6) incubated in 0.05 mg/mL 3,3'-diaminobenzidine (Sigma), 0.25% nickel ammonium sulphate (BDH, Poole, UK) in 0.05 M Tris-HCL (pH 7.6) containing 0.01%  $H_2O_2$  (Merck) for 15 min; 7) washed in aquadest for 10 min; 8) dehydrated in ethanol; and 9) mounted in Entellan.

### Morphometry

Every 50th section stained for SOM along the rostro-caudal axis of the BSTc on one side of the brain (22) was used for analysis with the help of a specially developed program on an IBAS (Kontron Elektronik, Munich, Germany) image analysis system. The image analysis system was connected to a scanning stage control box (MCU, Carl Zeiss, Oberkochen, Germany) and had a Sony B/W CCD-camera for image acquisition. Both the scanning stage and the camera were mounted on a microscope (Carl Zeiss) equipped with planapo objectives. To provide optimal contrast and homogenous illumination of the section the voltage of the light source was set maximally. The light was reduced by neutral gray filters (0.03/0.12/0.5/Schott; Mainz, Germany) to improve light contrast. For each section, the analysis consisted of the following steps:

By using the plan  $\times 2.5$  objective of the microscope, a low magnification image covering the BSTc area was obtained and loaded into the IBAS image memory.

In this image the BSTc was outlined manually on the basis of the distribution of the SOM immunoreactivity in neurons and fibers (see figure 86.3). Subsequently, the image analyzer covered the outlined area with a grid of rectangular fields, each with the size of the area

displayed by the camera when the  $\times 40$  objective was installed.

By a random systematic sampling procedure, 50% of the fields (which were for at least 80% covered by the outlined area) were selected for analysis. Taking into account the aberration of the optical axis between the  $\times 2.5$  and the  $\times 40$  objective, the pixel positions of the selected rectangular fields in the 2.5 image were converted into scanning stage coordinates to position the corresponding areas of the BSTc in front of the camera when using the  $\times 40$  objective.

After the  $\times 40$  objective was installed, the image analyzer moved the scanning stage automatically to the coordinates of the selected fields. In each field, SOM-positive neurons containing a nucleolus were counted manually, taking into account the exclusion lines according to Gundersen (26). Neurons with double nucleoli were never seen. The spectrum of neuronal sizes was equally distributed among the different groups.

The total volume of the BSTc was calculated by rostro-caudal integration of the outlined areas, taking into account the distance between the measured sections. The neuronal density was calculated on the basis of the nucleolus counts in the sample volume. An estimation of the total number of SOM neurons was obtained by multiplying the total volume with the mean neuronal density. The finding that the mean BSTc volumes of the various groups are almost twice as large as those found in the study of Zhou et al. (22) can be explained by the fact that in the present study another peptidergic system (SOM instead of vasoactive intestinal polypeptide) was used as a marker and also an antigen retrieval technique (i.e. microwave tissue pretreatment), which makes the staining more sensitive (24, 27).

### Statistics

Differences among the groups were statistically evaluated by the nonparametric Kruskal-Wallis multiple comparison test. Differences between the groups were analyzed two-tailed using the Mann-Whitney U test with a 5% experiment wise error rate (sequential Bonferroni method). Throughout this study values are expressed as mean  $\pm$  SEM. A significance level of 5% was used in all statistical tests.

### Results

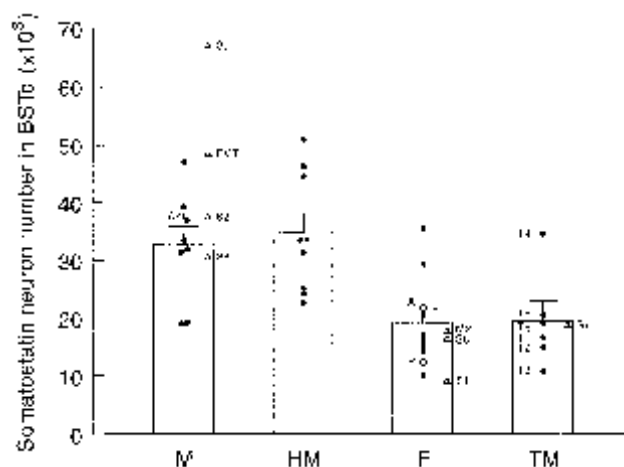
Differences among the groups were statistically significant by the nonparametric Kruskal-Wallis multiple comparison test ( $P = 0.002$  for SOM neuron number). No statistical group differences were found for age ( $P = 0.090$ ), brain weight ( $P = 0.125$ ), postmortem

time ( $P = 0.738$ ), fixation time ( $P = 0.065$ ), or storage time ( $P = 0.308$ ). To further test whether the differences in the BSTc between the groups were affected by possible confounding factors, such as paraffin-embedded storage time of sections, fixation time, postmortem time, or brain weight, an analysis of covariance was carried out. These factors seemed to have no significant effect on the BSTc SOM neuron numbers ( $P > 0.10$ ).

The number of SOM neurons in the BSTc of heterosexual men ( $32.9 \pm 3.0 \times 10^3$ ) was 71% higher than that in heterosexual women ( $19.2 \pm 2.5 \times 10^3$ ) ( $P < 0.006$ ), whereas the number of neurons in heterosexual and homosexual men ( $34.6 \pm 3.4 \times 10^3$ ) was similar ( $P < 0.83$ ). The BSTc number of neurons was 81% higher in homosexual men than in heterosexual women ( $P < 0.004$ ). The number of neurons in the BSTc of male-to-female transsexuals was similar to that of females ( $19.6 \pm 3.3 \times 10^3$ ) ( $P = 0.83$ ) (see also figures 86.1 and 86.2). In addition, the neuron number of the FMT was clearly in the male range (see figure 86.1). The number of neurons in transsexuals was 40% lower than that found in the heterosexual reference males ( $P < 0.04$ ; see the legend to figure 86.1) and 44% lower than that found in the homosexual males ( $P < 0.02$ ). Including patients S2, S3, and S5 in the male group and S1, S6, and M2 in the female group or S7 in the transsexual group to increase the number of their respective gender groups enhanced the level of significance among the groups ( $P < 0.001$  for SOM neuron number). There seemed to be no clear difference in the BSTc number of neurons between early-onset (T2, T5, T6) and late-onset transsexuals (T1, T3), indicating that their smaller number of neurons is related to the gender identity per se rather than to the age at which it became apparent. No indication was found for a relationship between cause of death and BSTc neuron numbers. Analysis of the BSTc volumes showed a similar pattern of differences among the groups with heterosexual men having a BSTc volume of  $4.60 \pm 0.28 \text{ mm}^3$ , similar to that in homosexual men ( $5.00 \pm 0.39 \text{ mm}^3$ ) ( $P = 0.76$ ). The BSTc volume of females ( $3.38 \pm 0.41 \text{ mm}^3$ ) and that of transsexuals ( $3.58 \pm 0.19 \text{ mm}^3$ ) did not differ either ( $P = 0.50$ ). The volumes of all males, regardless of sexual orientation, vs. all females or vs. all genetic male transsexuals were statistically highly significant ( $P \leq 0.01$ ). The FMT had a BSTc volume in the male range ( $4.80 \text{ mm}^3$ ).

## Discussion

In the present study, we show regardless of sexual orientation: 1) a sex difference in SOM neuron numbers in the human BSTc, with males having almost twice as

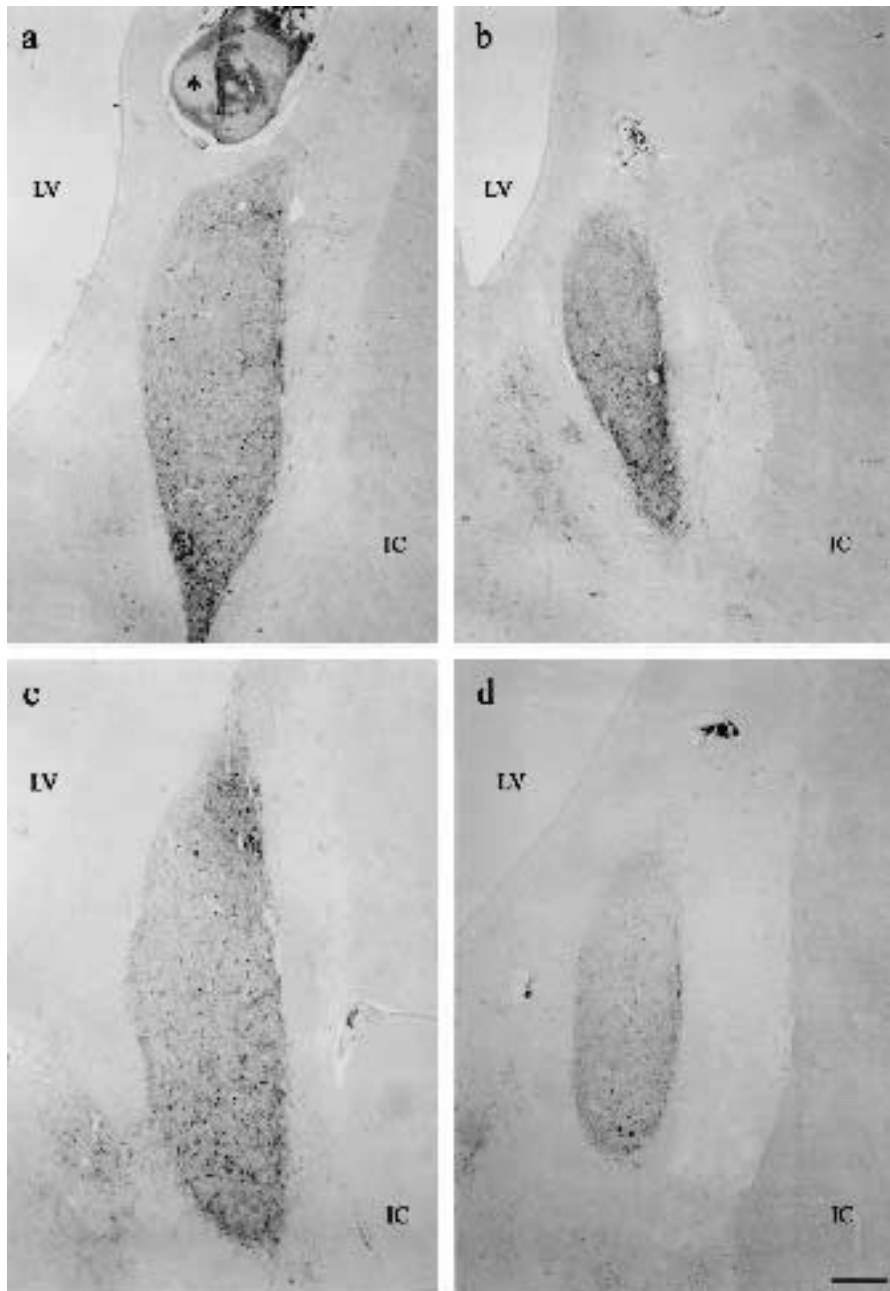


**Figure 86.1**

BSTc neuron numbers. Distribution of the BSTc neuron numbers among the different groups according to sex, sexual orientation, and gender identity. M, Heterosexual male reference group; HM, homosexual male group; F, female group; TM, male-to-female transsexuals. The sex hormone disorder patients S1, S2, S3, S5, S6, and M2 indicate that changes in sex hormone levels in adulthood do not change the neuron numbers of the BSTc. The difference between the M and the TM group ( $P < 0.04$ ) is also statistically significant according to the sequential Bonferroni method if S2, S3, and S5 are included in the M group or if S7 is included in the TM group ( $P \leq 0.01$ ). Note that the number of neurons of the FMT is fully within the male range. Whether the transsexuals were male oriented (T1, T6), female oriented (T2, T3, T5), or both (T4) did not have any relationship with the neuron number of the BSTc. The same holds true for heterosexual and homosexual men. This shows that the BSTc number of somatostatin neurons is not related to sexual orientation. A, AIDS patient. The BSTc number of neurons in the heterosexual man and woman with AIDS remained well within the corresponding reference group (see figure 86.1), so AIDS did not seem to affect the somatostatin neuron numbers in the BSTc. P, Postmenopausal woman. S1 (♀ 46 yr of age): adrenal cortex tumor for more than 1 yr, causing high cortisol, androstendione, and testosterone levels. S2 (♂ 31 yr of age): feminizing adrenal tumor that induced high blood levels of oestrogens. S3 (♂ 67 yr of age): prostate carcinoma; orchiectomy 3 months before death. S5 (♂ 86 yr of age): prostate carcinoma; prostatectomy; orchiectomy, and antiandrogen treatment for the last 2 yr. S6 (♀ 25 yr of age): Turner syndrome (45,X0; ovarian hypoplasia). M2 (♀ 73 yr of age): postmenopausal status.

many SOM neurons as females; 2) a number of SOM neurons in the BSTc of male-to-female transsexuals in the female range; and 3) an opposite pattern in the BSTc of a female-to-male transsexual with a SOM neuron number in the male range.

Analysis of the total number of SOM neurons of the human BSTc in individual patients with highly different hormone levels does not give any indication that changes in sex hormone levels in adulthood change the neuron numbers. Because the transsexuals had all been treated with estrogens, at least for some time (see table 86.2), the reduced neuron numbers of the BSTc could theoretically be due to the presence of high levels of circulating estrogens. Arguments against this possibility come from the finding that transsexuals T2 and T3

**Figure 86.2**

Representative immunocytochemical stainings of the somatostatin neurons and fibers in the BSTc of a reference man (*a*), reference woman (*b*), homosexual man (*c*), and male-to-female transsexual (*d*). Note the sex difference regardless of sexual orientation. The male-to-female transsexual has a BSTc in the female range. \*, Blood vessel. *Bar* represents 0.35 mm.

**Table 86.2**  
Clinicopathological data of subjects with gender identity disorder

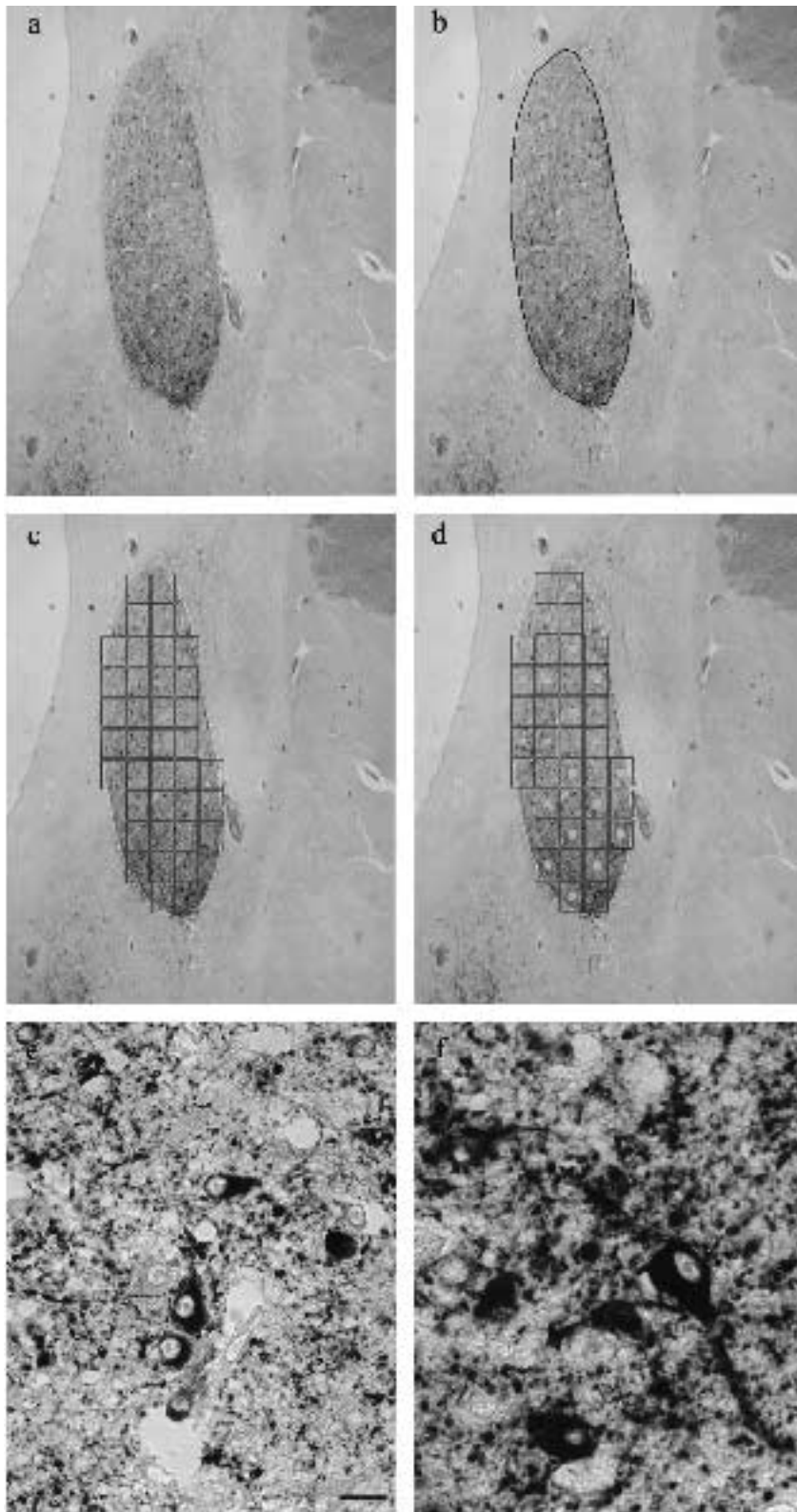
Patient No. (NBB)	Age (yr)	Age of Hormonal Treatment/Orchiectomy	Hormone Treatment	Cause of Death
<i>Male-to-female transsexuals (n = 7)</i>				
T1 (84020)	50	42/44	Age 42: Stilbestrol 5 mg 1 dd; after 2 months to 5 mg 2 dd; age 44: CPA 50 mg 2 dd; (treatment lasted 4 yr, stopped 2 yr before death); Ethinyloestradiol 50 µg 2 dd (treatment lasted 8 yr until death)	Suicide
T2 (84037)	44	35/37	Age 35: stilbestrol 5 mg 3 dd; after 2 months to 5 mg 2 dd; CPA 50 mg 1 dd; 1977: CPA 50 mg 2 dd; stilbestrol 5 mg 1–2 dd (generally this lasted 7 yr until death; stilbestrol stopped about 15 months before death)	Cardiovascular death
T3 (88064)	43	36/39	Age 36: received standard CPA treatment (50 mg 2 dd) until 2 yr before death; At age 39 received standard ethinyloestradiol treatment (50 µg 2 dd) that stopped 3 months before death	Sarcoma, right-side temporal
T4 (93042)	36	NA/no orchiectomy, testes atrophy	CPA 50 mg 1 dd at least the last 10 months before death; the patient received estradiol in combination with hydroxyprogesterone in therapeutical dosages. Exact period of treatment is not known but based on the significant testes atrophy she was most probably treated for a period of about 5 yr or more.	AIDS, pneumonia, pericarditis, cytomegaly in brain
T5 (93070)	53	40/50	Age 40: stilbestrol treatment (stopped after 1 yr); at age 43–47: premarin 0.625 mg dd; at age 47–50: premarin 3.75 mg dd; at age 50–53: premarin 2.5 mg 3 dd; CPA 50 mg 1 dd; topical estrogen cream (estrogen treatment stopped 3 months before death)	Acute fatty liver due to alcohol abuse
T6 (95018)	48	35/36	Age 35: spironolactone 100 mg 2 dd; CPA 50 mg 2 dd; ethinyloestradiol 50 µg 2 dd; at age 36–40: CPA 50 mg 2 dd; ethinyloestradiol 50 µg 2 dd; at age 40–48; aldosterone 100 mg 1 dd; ethinyloestradiol 50 µg 1 dd (treatment lasted until death)	Cardiovascular death
S7 (96088)	84	No orchiectomy or sex reassignment therapy	Patient did not receive sex hormone replacement therapy	Lung carcinoma
<i>FMT (n = 1)</i>				
FMT (98138)	51	27/28	Age 27: testosterone sustanon 250 mg, twice a month injections; at age 30 testosterone undecanoat 40 mg 3 dd. At age 34 testosterone undecanoat 40 mg 2 dd; At age 36 testosterone undecanoat 40 mg 4 dd; At age 44 testosterone sustanon 250, twice a month injections; At age 47 to 48: testosterone sustanon 250, every 3 weeks; from the age 48 until the age of death (51), no testosterone replacement therapy anymore	Cachexia

NBB, Patient number of the Netherlands Brain Bank; CPA, cyproterone acetate; NA, not available; AIDS, acquired immune deficiency syndrome.

both showed a small BSTc (figure 86.1), despite the fact that T2 stopped taking estrogens about 15 months before her death because of hyperprolactinemia, and T3 no longer received hormone treatment when a sarcoma was found about 3 months before she died. T5 continued to take estrogens until 3 months before death and had even more SOM neurons than T3, whereas T1 and T6 continued to take estrogens until death and even had higher SOM neuron numbers than T2 and T3 (figure 86.1). Furthermore, a 31-yr-old man (S2), who suffered for at least 1 yr from a feminizing adrenal tumor that produced high blood levels of estrogens, still had a BSTc neuron number in the normal male range (the latest highest serum estradiol levels be-

fore death varied between 577–779 pmol/L; the normal range is 50–200 pmol/L).

Our results might theoretically also be explained by a lack of androgens in the transsexual group because all subjects, except for T4, had been orchiectomized. We, therefore, studied two nontranssexual men (S3 and S5) who had been orchiectomized because of prostate cancer 3 months and 2 yr before death, respectively, and found that the BSTc neuron number of S3 was close to the mean of the male group and that the BSTc number of neurons of S5 was even the highest observed (figure 86.1), indicating that orchiectomy did not cause any decrease in SOM neuron numbers. Not only were five of the transsexuals orchiectomized, they all used



**Figure 86.3**

The image analysis procedure. *a*, Illustration of a somatostatin immunoreactive BSTc. *b*, The BSTc is outlined manually. *c*, Outlined BSTc is divided automatically into rectangular fields. *d*, Fifty percent of the fields is selected by a random systematic sampling procedure. *e*, Higher magnification of somatostatin neurons in a field displayed by the camera when the  $\times 40$  objective is installed. Only somatostatin-positive neurons with a visible nucleolus were counted (see *Morphometry in Materials and Methods*). Bar represents 40  $\mu\text{m}$ . *f*, Example of a clearly visible nucleolus in a somatostatin immunoreactive neuron.

the antiandrogen cyproterone acetate (CPA). However, an effect of CPA reducing the number of SOM neurons of the BSTc is highly unlikely because S5 had taken CPA during the last 2 yr of his life and his BSTc neuron number was at the upper end of the male range, whereas T6 had not taken CPA for the past 10 yr, and T3 took no CPA during the last 2 yr before her death, and they still had relatively low numbers of SOM neurons.

The BSTc SOM neuron numbers of two postmenopausal women [73- (M2) and 53-yr-old (P)] and of a 25-yr-old woman with Turner syndrome (S6: complete 45,X0, with ovarian hypoplasia) were completely within the normal female range (figure 86.1). If high estrogen levels would have a reducing effect on BSTc neuron numbers, the opposite effect (high neuron numbers) would be expected in the postmenopausal women and the Turner syndrome patient due to their low endogenous sex hormone level status. However, this was not the case. Noteworthy is that according to the available clinical data the two postmenopausal women did not receive any estrogen replacement therapy either. Although the Turner syndrome patient had been receiving hormone replacement therapy since she was 16 yr of age, her neuron numbers were even higher than P, whereas she had almost the same BSTc neuron number as M2 who did not receive such a therapy. Again, this argues against the probability of an estrogen-induced reduction effect on the number of SOM neurons. Finally, the BSTc neuron number of a 46-yr-old woman who had suffered for at least 1 yr from a virilizing tumor of the adrenal cortex (that produced very high blood levels of androstendione and testosterone) was also clearly within the lower spectrum of that of other women (figure 86.1; S1: latest androstendione serum level before death was 48.0 ng/mL; the normal range for women is 0.4–3.5 ng/mL; the latest serum testosterone level before death was 26.82 nm/L; the normal range for women is 1.04–3.30 nm/L). Thus, an increasing effect of testosterone on the BSTc neurons does not seem likely to be the case either. Furthermore, it should be noted that the FMT stopped taking testosterone 3 yr before death while having a BSTc neuron number clearly within the male range.

In conclusion, estrogen treatment, orchiectomy, CPA treatment, or hormonal changes in adulthood did not show any clear relationship with the BSTc SOM neuron number. In addition, we had the unique opportunity to study the brain of an 84-yr-old man (S7) who also had very strong cross-gender identity feelings but was never orchiectomized, sex re-assigned, or treated with CPA or estrogens. Interestingly, this man had also a low BSTc SOM neuron number that was fully in the female range (see figure 86.1, S7). This case pro-

vides an additional argument against the view that orchiectomy, CPA, or adult estrogen treatment of the transsexuals would be responsible for the reduced somatostatinergic neuron numbers. Moreover, studies that investigated the effects of estrogen treatment on hypothalamic SOM neurons in (castrated) rats are also not in support of such an effect. Estrogen treatment does not reduce the amount of SOM messenger RNA (mRNA) in neurons but even enhances its neuronal expression (28). Moreover, another animal study indicates that, although changes occur in the hypothalamic neuronal expression of SOM mRNA due to castration or testosterone treatment of male rats, no differences in hypothalamic SOM neuron numbers are induced at all by either of such treatments (29). This observation is also in agreement with the control SOM neuron numbers of the castrated male patients (S3, S5) and testosterone-exposed (S1) female patient. Together, all these data clearly indicate that sex hormone-mediated reduction (or enhancement) effects on transsexual BSTc neurons in adulthood are extremely unlikely to be the underlying mechanism of the observed somatostatinergic BSTc differences.

In short, our findings seem to support the hypothesis that the somatostatinergic sex differences, the female number of SOM neurons in the BSTc of the male-to-female transsexual brain and the male number of SOM neurons in the BSTc of the FMT are not the result of changes of sex hormone levels in adulthood. Instead, the neuronal differences are likely to have been established earlier during development [see also Zhou et al. (22), and for functional differences see Cohen-Kettenis et al. (30)]. In line with this reasoning are the developmental data on the rat BST showing that adult volumes and neuron numbers of BST subdivisions are orchestrated by androgen exposure during early brain development (31, 32). Such a mechanism is also in agreement with data of Breedlove (33, 34) showing that perinatal androgens but not adult variations in androgen exposure induce differences in the total neuron number of the rat spinal nucleus bulbocavernosus. Apart from such well known irreversible “organizing” effects of sex hormones on the developing brain, the possibility of a direct action of genetic factors on sexual differentiation of the brain should not be ruled out (35).

We are aware of the fact that our data are based on postmortem brain material derived from a heterogeneous patient population of which each individual's clinical status might have had an impact on the brain. However, despite that we were still able to find striking sexual dimorphic differences (that become even more significant if patients S1, S2, S3, S5, S6, S7, and M2 are included in their respective gender groups; see statistics and the legend to figure 86.1). An exciting addi-

tional new finding came from the FMT who revealed a "masculine" BSTc, which is completely in line with the sexual brain paradigm (7, 22, 30, 36–40).

Although our collection of male-to-female transsexual brains is small, it offers new opportunities to explore neurobiological correlates of transsexualism, as has previously been done in relation to sexual orientation (4–6). The development of high resolution imaging techniques may allow in vivo volume measurements of particular brain areas in much larger groups of transsexuals, which could extend our findings in the distant future. Although brain imaging proved to be useful in visualizing [e.g. septo-hypothalamic brain injuries leading to hypersexuality or altered sexual preference (9, 10)], precise neuroanatomical delineation of small brain structures such as the BSTc or neuronal counts are, at present, not possible using such techniques.

Taking into account the aforementioned limitations of our studies, the present study of SOM neurons in the human BSTc provides unequivocal new data supporting the view that transsexualism may reflect a form of brain hermaphroditism such that this limbic nucleus itself is structurally sexually differentiated opposite to the transsexual's genetic and genital sex. It is conceivable that this dichotomy is just the tip of the iceberg and holds also true for many other sexually dimorphic brain areas.

Because the sexually differentiated brain in general (41) may be the basis of sex differences in the prevalence of many neurobiological diseases and disorders (7), more studies are needed to further unravel the potential determinants of the sexual dimorphic brain and its related clinical disorders.

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## VI EPILOGUE

At the close of our journey it is worthwhile to cast an eye back from whence we have come. In this respect, Frank Beach's own review of the field is fitting. It is encyclopedic, with a bibliography that spans the centuries and continents over which endocrine research took place. At times a bit pedantic, once completed there is no doubt that origins of endocrinology and its effects on behavior have been thoroughly exposed from the development of nonhuman animal models to human behavior.

In this selection, Beach designates three eras of behavioral endocrinology research:

1. *The Predisciplinary Era: 1849–1900* This is the period of an experiment here, an experiment there, but before the discipline of endocrinology is born as a real science.
2. *The Formative Era: 1900–1950* This era marks the concerted development of behavioral studies.
3. *The Modern Era: 1950–1981* The year 1981 was chosen because it is the date of Beach's review. This era is marked by the development of true specialists in behavioral endocrinology and an accelerating rate of increase in empirical evidence.

One might suggest that with the publication of the first two papers of this volume by Goy and McEwen (1980), the field of Hormones and Behavior entered a fourth era, which might be called *the Contemporary Era*. What we see in this volume is the movement of the field to both ends of the empirical spectrum—at one end, human behavior and at the other, cellular behavior. These two poles of the research continuum chronicle sex differences in brain activity at one end and the molecular action of steroid hormones and their receptors, at the other.

What has driven the formation of these eras beside simple human curiosity? Why is our own era focused on one's identity as male or female, gay or straight, gendered or transgendered on the one hand and the molecular actions of estrogens and their receptors on the other?

One powerful force is money and funding. So it is interesting to read in Beach that *the Modern Era* is

marked by the development of formal funding mechanisms by the National Research Council's Committee for Research in the Problems of Sex. What is most interesting in Beach's account is that this committee was conceived of as a body to solve the problems of *prostitution* and *venereal disease*. In spite of the fact that Beach recounts an internally mediated shift by the Committee to fund empirical research on the physiology and psychology of *reproductive* behavior, it is worthwhile considering how the perception of science as solving what is considered as non-normative behavior shapes the design and interpretation of experiments described in the chapters of this volume.

Another force is changing mores. The work of Beach and his colleagues (including Kinsey) opened up the empirical study of sex in all its forms. Human sexual behavior is far more acceptable to study now than in Beach's day. Thus, changing mores has allowed the exploration of the variation described in the *Formative Era*. New technologies make it possible to study the mechanisms underlying that variation in humans. Because of the divergence to the far poles of the empirical spectrum, care needs to be taken not to apply whole cloth assumptions based on rodent models. Humans are not rodents and, as the first selections by Beach demonstrated rodent "sexuality" is not as simple as the interpretations over the experimental eras have suggested.

It could be argued that studying human behavior at one end and molecules at the other represents the splintering of a once coherent field. However it might also be viewed as an attempt to form new disciplines. Beach, himself, pointed out that even in the *Modern Era* disciplinary lines were fading:

Scientists with their doctorates in psychology study development of progesterone receptors in neurons of the rat hypothalamus while other investigators initially trained in pharmacology invent elegant behavioral measures of sexual motivation in the estrous female. These developments appear to represent more than a mere borrowing of techniques...they seem to reflect progress toward recognition of common goals and shared theoretical interests. (p. 370)

Perhaps because of this mixing up of methodologies and perspectives, bit-by-bit the *Modern Era* has provided the methodologies for experiments that would actually reveal the breadth of the human behavioral spectrum. Perhaps an exploration of the diversities and range of sexual behaviors beyond the paradigms of “masculinization/defeminization.” One frontier that has yet to be thoroughly explored is the cerebral cortex’s involvement in sexual behavior—“cognition” not sex is what comes to mind about the neocortex and “sex”—even our sexual identities—seems remotely related to cognition; however, experience’s effects on the neocortex are by now well-documented, and there is likely not one neuron in the brain that is not affected by steroid hormones either directly or indirectly. So the cerebral cortex is the integrator of hormones and behavior par excellence. Thus, it may be a site worth studying in more detail with respect not to sex differences but with respect to sexual diversities.

In this sense, Beach’s account of how he, himself, became involved in the search for an understanding of the interplay between hormones and behavior supports the importance of the neocortex in sexual behaviors:

The present writer’s involvement in research on hormones and behavior began in 1936 during a post doctoral year in the laboratory of K. S. Lashley at Harvard University. It originated as the result of conversation with graduate students in the adjoining endocrinology laboratory of F. L. Hisaw. The author’s experiments had shown that male rats ceased copulating after infliction of large lesions to the cerebral cortex, and Hisaw’s students pointed out that brain injury might indirectly reduce testosterone secretion by altering pituitary function. To check this possibility brain-operated rats that had ceased to mate were injected with large amounts of testosterone, and some then began to copulate.

Postmortem examination of the testes and accessory glands proved that testosterone secretion had not been impaired despite the cerebral lesions. Therefore it appeared that supplying very large amounts of exogenous androgen had somehow reversed a behavioral deficit caused by CNS injury. This serendipitous finding launched a career of research on the hormonal control of behavior. (pp. 361–362)

By Beach’s account what interrupted sexual behavior in these rodents were the large lesions in the *cerebral cortex*. Thus, at a very early stage in his career, by interacting broadly with his colleagues, Beach spanned the disciplines of behavior, endocrinology, and brain science to demonstrate that sex is not a unitary behavior or a simple reflex. It is infinitely complex involving not only the hypothalamus, pituitary, and testes/ovaries but the cerebral cortex, the cognitive brain.

Another frontier to explore is the effect of experience on the brain regions that are classically considered to mediate sex such as those which have not traditionally been considered plastic. Such an inquiry might begin to merge more contemporary understandings of how

nurture influences nature supporting and drawing on disciplines from outside of science such as feminist critiques of essentializing the body. As well, a source for further elucidation into the range of sexual behaviors would be cross cultural studies of how non-Euro-American sexual traditions shape the central nervous system.

If the *Contemporary Era* spans 1980–2006, ask yourself, What will we understand at the end of the next 30–50 years of inquiry?

Behavioral endocrinology has been described as “an emerging discipline” (Beach, 1975), and this is at least partly accurate because the amount of research on hormonal control of behavior has increased 10- to 20-fold in the last 25 years. Furthermore the majority of individuals now engaging in such research are young men and women whose postgraduate degrees were awarded after 1960–1965. The necessity of “keeping up with” current scientific literature plus a desirable preoccupation with immediate and forthcoming research problems leave little time for contemplation of historical aspects of the field. The present article has been written because of a conviction that historical perspective is important in any science and has particular value for younger contributors in an area of investigation that currently is undergoing rapid expansion.

In their excellent textbook, *General Endocrinology*, C. D. Turner and J. T. Bagnara (1976) divide the development of that discipline into three main periods. (1) First came its “early history,” which stretched from the ancient Greeks to the end of the 19th century. (2) Next was its “birth as a real science,” which was signaled by Bayliss and Starling’s discovery of the hormone secretin and their demonstration of its manner of action. Parturition was followed by an era of “slow growth of the infant science.” (3) The most recent era is defined as one of spectacular growth and is described as follows.

The 1940’s ushered in a period of biochemical expansion and inquiry that was unprecedented in the history of endocrinology.... Happily, the tremendous impact of biochemistry, particularly with reference to intracellular controls and gene activity, felt with increasing strength during the past few decades, has led to a reorientation of thinking. With the perfusion of pure science techniques and concepts into all phases of the subject, endocrinology has become a respected science and a dignified field of research specialization.

Turner and Bagnara’s analysis and evaluation provide a useful and edifying framework for comparison with historical developments in the study of hormonal effects on behavior. This history also can be divided into three parts.

1. The first might be termed the *predisciplinary era* because it represents a time before there was a discipline of endocrinology. For reasons to be explained later its beginning is unequivocally set at 1849. We will designate the end of this era as 1900 simply because this is when Turner and Bagnara say endocrinology was born as a “real science.”

2. The second phase in our history of behavioral studies will be defined as the *formative era*. It lasted approximately 50 years, although choice of a terminating date is purely arbitrary and it could be set almost anywhere between 1950 and 1960. Some feeling for a change in the rate of development of behavioral studies can be gained from the chronological record of books and review articles published prior to 1955.

An extremely influential compendium marking progress in the development of endocrinology was the first edition of *Sex and Internal Secretions* which appeared in 1922 under the editorship of Edgar Allen and sponsorship of the National Research Council for Research in Problems of Sex (a body that will be described later). This edition comprised 912 pages with 19 different chapters on endocrine functions of various glands and one chapter titled “Sex Drive” which dealt with behavior and was written by the psychologist C. P. Stone. In terms of pages occupied, Stone’s chapter represented 5% of the total. The second edition appeared in 1932, and although the total length had expanded to 1345 pages and 24 chapters, Stone’s single chapter on behavior still represented only 4% of the total. The third edition appeared in 1961 under the editorship of W. C. Young. There were 24 chapters, 6 of which dealt with behavior and occupied 21% of the total number of pages. Differences in emphasis between the 1932 and the 1961 editions undoubtedly reflected different editorial policies and interests, but most importantly they indicate a major increase in the amount of behavioral research published during the intervening 29 years.

Other landmark publications appearing during or soon after the 1940s reflected a fairly rapid growth and consolidation of research on hormones and behavior. Noteworthy examples include an extensive review

article by W. C. Young which appeared in 1941 in the *Quarterly Review of Biology* under the title, "Observations and Experiments on Mating Behavior of Female Mammals." In 1948 the present writer's book, *Hormones and Behavior*, represented the first attempt to survey the entire field. In 1950 a comprehensive *Symposium on Steroid Hormones* edited by E. S. Gordon contained an extensive review by N. E. Collias titled "Hormones and Behavior with Special Reference to Birds and the Mechanism of Hormone Action." In 1952 there appeared the results of an International Ciba Foundation Colloquium on Endocrinology with the subtitle *Hormones, Psychology and Behaviour and Steroid Hormone Administration*.

A selected list of participants named in alphabetical order included F. A. Beach, E. W. Dempsey, T. R. Forbes, G. W. Harris, M. Klein, A. S. Parkes, C. P. Richter, and S. Zuckerman. Close perusal of this little volume shows concern with and beginnings of progress in study of many issues at the forefront of today's research enterprise. For example, in discussion of one of the major papers G. W. Harris mentions preliminary results from his own laboratory indicating that sexual receptivity may be induced in spayed rabbits if estrogenic hormone is applied directly to particular regions of the brain. Other speakers describe research on species differences in behavioral effects of steroids, influence of hormones on learned responses, "psychological aberrations" associated with endocrine disturbance, and mechanisms of hormone action on behavior.

Clearly, by the middle of the present century study of hormonal control of behavior had become a separately identifiable research specialty even though very few scientists were specialists in the sense of concentrating exclusively or even primarily on behavioral problems. At this point the formative period ends, or more accurately begins to merge with the third and current era.

3. The *modern* era has already lasted 25–30 years and has been marked by development of true specialists in behavioral endocrinology as well as by a positively accelerated rate of increase in empirical evidence provided by invention of new methodologies. Status as a mature discipline has not yet been achieved but this seems very likely to occur within the next quarter century.

The following account deals almost exclusively with predisciplinary and formative periods even though some of the most outstanding and exciting developments in behavioral endocrinology have come during the modern era. At several points it will prove necessary to violate our self-imposed limits, but the primary objective of this account is to set modern day research in long-term perspective. It is instructive to discover that some of the major questions under examination

today were first asked more than 100 years ago. Various theories currently being tested were initially enunciated in the 19th century. Unless a scientist is familiar with the history of his subject he can neither avoid the errors of his predecessors nor profit fully from their successes.

The philosopher Whitehead once observed that the last thing to be discovered in any science is what that science is all about. Investigators presently active in research on hormones and behavior may achieve some appreciation of what behavioral endocrinology is all about if they learn something of its origins and earliest periods of development.

### Nineteenth-Century Beginnings of Behavioral Research

In the second half of the last century, before there was endocrinology, and therefore before anyone could be designated an endocrinologist, there was nevertheless a growing appreciation of the importance of what we now term "endocrine glands" or "glands of internal secretion." Not all of the glands had been identified, and many of their functions were only guessed at, but one fact is particularly relevant to this history. The term "function" was used to refer to behavioral or psychological characteristics as well as morphological and physiological features of the organism under consideration. For example, in 1883 when the Swiss surgeons T. Kocher and E. Reverdine described the symptoms of myxedema caused by thyroid insufficiency they stressed especially the patients' emotional apathy and intellectual deterioration. Eight years later, when G. R. Murray successfully treated myxedema by administering an emulsion of sheep thyroid, the psychological improvement and personality changes were regarded as important aspects of the medication's effects.

Investigations into what was to become endocrinology were carried out by practicing physicians and by laboratory experimentalists. The science of physiology was in its ascendancy and most experimental workers were trained as physiologists and anatomists. Most held medical degrees, and a few even combined animal experimentation with an active medical practice. When it came to studying effects of internal secretions on animals, behavioral consequences of experimental manipulations were considered just as important as effects of any other type, and their occurrence was never neglected.

### Animal Experimentation in the Predisciplinary Era

A single study published in 1849 by A. A. Berthold, Professor of Physiology at the University of Göttingen, has been defined as "the first proof of endocrine function as we know it" (Forbes, 1949). Berthold castrated six roosters and in three of them returned one of the

severed testicles to the bird's abdomen where it could establish a new blood supply and survive. The three castrates without implanted testes became ordinary capons, but Berthold's description of the three experimental individuals is a milestone in the history of behavioral endocrinology.

So far as voice, sexual urge, belligerence, and growth of comb and wattles are concerned, such birds remained true cockerels. Since, however, transplanted testes are no longer connected with their original innervation, and since...no specific secretory nerves are present, it follows that the results in question are determined by the productive functions of the testes, i.e., by their action on the blood stream, and then by corresponding reaction of the blood on the entire organism...of which...the nervous system represents a considerable part. (Forbes, 1949)

Two aspects of these findings and their interpretation deserve emphasis. (1) Three of four characteristics mentioned as proof of the "productive function of the testes" are behavioral, i.e., crowing, mating, and fighting; and thus, "the first proof of endocrine function as we know it" was actually an experiment in behavioral endocrinology. (2) Berthold's explanation was that some testicular substance is transported by the blood and produces a reaction in the nervous system; and this interpretation dominates modern day theories of hormonal action on behavior.

During the half century that followed Berthold's discovery physiologists in France, Italy, Germany, and Russia undertook investigation of relations between mating behavior of various animals and functions of the sex glands. Castration had long been known to eliminate the "sexual instinct"; but emergence of the concept of internal secretions raised questions as to how they might affect behavior. There was general agreement that the final locus of action had to be the nervous system, but whether the action was direct or indirect remained a matter of dispute.

One very influential proponent of a "peripheralistic" explanation was the Russian physiologist, J. R. Tarchanoff, whose research dealt with the seasonal onset of copulatory behavior in male frogs. Tarchanoff observed that before mating begins the frog's seminal vesicles "increase from the size of an apple pip to that of a wild strawberry." He suggested therefore that sensory impulses arising in the distended vesicles are transmitted to special centers in the amphibian's brain where they give rise to tendencies to pursue and mate with gravid females. Tarchanoff explained that a male castrated before onset of the breeding season does not mate because the sex accessories do not hypertrophy, and the brain is thereby deprived of the essential peripheral stimuli (Tarchanoff, 1887).

Tarchanoff's theory was put to experimental test by an Austrian physiologist, Eugen Steinach, who re-

moved the frog's seminal vesicles but left the testes intact, and showed that the operation had no deleterious effects on sexual performance (Steinach, 1894). Steinach was Professor and Director of the Biological Research Institute of the Academy of Sciences in Vienna. In this capacity he conducted many important studies on hormones and behavior (Beach, 1948). He was among the first to investigate behavioral and physiological effects of removing and then reimplanting the gonads of various species of amphibians, rodents, and birds. He attempted to induce precocious puberty by transplanting adult testes and ovaries into immature male and female animals. He conducted experiments on sex reversal by first removing the gonads and then implanting those of the opposite sex. Finally, he described the induction of "behavioral hermaphroditism," or bisexuality, in male and female rodents which had been hemicastrated and then implanted with one heterologous gonad.

Steinach's theory of hormone action was strictly "centralistic." He believed that sex hormones both stimulate and inhibit specialized centers in the brain, and possibly in the spinal cord. In 1863 I. M. Sechenov, Professor of Physiology at St. Petersburg University, had described cerebral inhibition of spinal reflexes in various animals. Following this lead in 1910 Steinach ablated parts of the medulla in male frogs out of breeding season and reported the postoperative appearance of the mating clasp despite the nonsecretory condition of the testes. He therefore concluded that when the testis is active (i.e., during breeding season) its secretions temporarily inhibit a critical center in the medulla and thus release the mating reflex.

#### Clinical Observations and Experiments on Human Subjects

During the half century we are now considering, a number of important clinical discoveries involving endocrine pathology were recorded. Mention has been made of successful treatment of myxedema with emulsions of sheep thyroid. In 1849 Thomas Addison described the disease, later named for him, which accompanies deterioration of the adrenal glands.

In textbooks on endocrinology it is obligatory to mention the name of Claude Bernard and he belongs in this history also. Eminent French physiologist and Professor of Medicine at the Collège de France, Bernard demonstrated the liver's release of sugar into the blood (1855) and coined the term "internal secretion" to describe this function. This can be slightly confusing to the present day student because strictly speaking sugar does not qualify as a hormone. Nonetheless, Bernard's reputation is well deserved for he also promulgated the concept of the internal environment and stressed the importance of its relative constancy to functional integrity of the organism.

Much more in the main line of endocrinological history was Bernard's brilliant successor, Charles Edward Brown-Séquard, whose scientific publications exceeded 500. Brown-Séquard's studies of adrenal function, which began in 1856, led him to state at the University of Paris in 1869, that "these glands have internal secretions and furnish to the blood useful if not essential principles" (Major, 1943).

It is unfortunate that Brown-Séquard's best remembered experiment is one he conducted in 1889 when he was 72 years old. Convinced of the broad and lasting importance of gonadal hormones, he attempted to achieve self-rejuvenation by 10 daily, intramuscular injections of a mixture containing blood from testicular veins, semen, and fluid from a crushed testis. This material was obtained from a dog (5 injections) and then from a guinea pig (5 injections). After several weeks of self-treatment Brown-Séquard recorded in his notebook an increase in muscular strength (measured with a dynamometer), improved bladder tonus (measured by the force with which urine was expelled), greater regularity of bowel function, and heightened resistance to mental fatigue (longer periods of uninterrupted writing and laboratory work).

Being neither a fool nor a charlatan, Brown-Séquard was alert to possible effects of autosuggestion. He therefore urged aging scientists of his acquaintance to repeat his experiments, and offered to provide the necessary material. There is no record of any acceptances, and instead Brown-Séquard's public announcement of his findings on June 1, 1889, was greeted with the sort of ridicule reflected in a review in the *Wiener medizinische Wochenschrift*.

Professor Brown-Séquard's audience appears to have received an impression of the intellectual capacity of the aged scientist very different from the one which he, in his elevated frame of mind, evidently expected to produce. This lecture must be regarded as further proof of the necessity of retiring professors who have attained their threescore years and ten. (transl. Steinach, 1940, p. 49)

One more attempt to reverse the effects of aging has importance for this history of early studies of hormones and behavior. This was an effort to induce in human patients certain supposedly beneficial results produced during experiments on several species of mammals. In the course of his wide-ranging program of animal studies, Eugen Steinach engaged in numerous attempts at rejuvenation, or, as he preferred to call it, "revitalization." After repeated experiments, he concluded that this could be achieved in male animals by the simple expedient of permanently occluding the vas deferens through which the testis discharges sperm. Choosing as subjects very old rats, dogs, and horses, Steinach securely tied off the vas on both sides, and found that after several weeks or months the animals

began to develop new hair growth which gave them a glossy coat, exhibited a much increased level of general activity, displayed heightened reactivity to various sorts of external stimulation, and showed "reawakened virility" when placed with females.

Microscopic examination of the testes of his subjects convinced Steinach that prolonged periods of vasoligation had produced two changes in the gonads. (1) The seminiferous tubules in which sperm are produced had degenerated and occupied much less space. (2) The interstitial tissue, containing Leydig cells that produce testosterone, had multiplied and comprised a proportionately increased component of the testicular mass. Finally, Steinach concluded that postoperative changes in appearance and behavior reflected an increased output of male hormone by the hypertrophied secretory portions of the sex gland.

Subsequent attempts by American scientists to replicate Steinach's experiments on rats proved unsuccessful, but before this occurred Steinach persuaded various urologists and surgeons to test the effects of vasoligation in elderly male patients. Dramatic reversal of the aging process was reported in many cases, and before the fashion had run its course literally thousands of men in Europe and America had been "Steinachized."<sup>1</sup> When the unreliability of positive results eventually became obvious the operation fell into disrepute, but by that time it was too late to save Steinach's status as a scientist, and financial support for his Institute in Vienna had been withdrawn.

It is worth repeating for emphasis that by the standards of his time Steinach's animal experiments were carefully conducted and that his theoretical concepts were highly original, some of them so much so that their final test was delayed for half a century until necessary technical advances had taken place. His total scientific output was prodigious and to the historian it is regrettable that Steinach's imaginative and largely fruitful endeavors have never been accorded the importance they deserve.

Like Brown-Séquard before him, Steinach was an honest investigator who misinterpreted some of his results (Price, 1975), but there are two important reasons for mentioning their errors in this historical account. (1) Such mistakes encouraged in the minds of many endocrinologists a conviction that psychological effects of hormones in humans could never be studied by genuinely scientific methods. (2) Even further, they laid grounds for a suspicion (later to become explicit) that even the behavior of lower animals is too labile and unpredictable to serve as a useful measure in endocrinological experimentation.

The first of these attitudes is reflected in statements such as those of Turner and Bagnara (1976) that early in the development of endocrinology "a clinical ap-

proach to the hormones made the subject particularly subject to quackery and led to considerable confusion," and that endocrinology became "a respected science and dignified field of research specialization [once it had been] perfused with pure-science techniques." The facts are, of course, that many theorists and experimentalists have always agreed that hormonal influences on human behavior not only exist, but can be studied scientifically, even though such research can be more difficult and complex than analysis of biochemical processes.

As early as 1892, in his classic work, *Psychopathia Sexualis*, Krafft-Ebing opined that sexual desire is related to "the physiological processes of the reproductive glands," although the degree of libido also is influenced by cerebral factors as evidenced by the persistence of sexual responsiveness in some postmenopausal women and male eunuchs. Krafft-Ebing believed in both central and peripheral effects, asserting that sensations from the reproductive organs aroused sexual ideas and images in the cerebral cortex.

Fortunately for the development of behavioral endocrinology, psychiatrists and psychologists have held fast to the conviction that hormones exert important effects on many functions which presently are too complex for study with "pure-science methods" as exemplified by biochemistry, but nevertheless can be examined objectively and quantitatively with eventual profit to our fuller understanding of behavior in both men and animals.

#### Initial Utilization and Subsequent Replacement of Behavioral Evidence in Endocrinology

According to Turner and Bagnara the years between 1900 and 1940 marked a period of slow growth of endocrinology. From the viewpoint of this history these decades were characterized by an initial phase in which endocrinologists made frequent use of behavioral evidence, followed by progressive increase in reliance on newly developed biochemical techniques which gradually reduced the use of behavioral tests, and eventually excluded studies of behavior from the mainstream of advance in the maturing discipline. It is not coincidental that the years between 1900 and 1950 can appropriately be designated as the *formative period* of behavioral endocrinology.

In 1917, at the Cornell Medical School, C. R. Stockard and G. N. Papanicolaou first described the "estrous cycle" in female guinea pigs, showing that different stages of development of the ovarian follicle are correlated with the presence of different kinds of cells in the vaginal mucosa. They employed the term "estrus" in its original sense which refers exclusively to behavior, and connotes the female's readiness to mate with males.

In 1922, two endocrinologists at the University of California in Berkeley, J. A. Long and H. M. Evans, described a similar vaginal cycle in female rats; and their validation of estrus in terms of behavior is revealed by the statement that its timing was determined by "offering individual females to a cage of males at 3-hr intervals when a record was kept of vaginal smears." The criteria for receptivity as a behavioral concept were very strict, and the detailed description of female mating reactions could scarcely be improved on today.

In 1923 Edgar Allen and Edward A. Doisy published their epochal paper, "An Ovarian Hormone: Preliminary Report of Its Localization, Extraction and Partial Purification, and Action in Test Animals." The hormone was estrogenic, and an important feature in its action on test animals was the behavior it evoked in female mice and rats.

While these spayed animals are in a condition of artificially induced estrus, they can be mated with normal males. They experience typical mating instincts, the spayed females taking the initiative in the courtship and showing no aversion to advances by the male. Since these animals will copulate only when in estrus, the conclusion seems justified that this follicular hormone is the cause of estrual or mating instincts.

At this time knowledge of pituitary function was so limited that in 1924 the fourth edition of Sir William Bayliss' standard text, *Essentials of Physiology*, devoted only two-thirds of a page to discussion of the gland. The situation was vastly improved after 1927 when Philip E. Smith of Columbia University devised an effective method of hypophysectomizing rats without producing excessive mortality. As evidence for completeness of his operations, Smith placed equal emphasis on degeneration of the ovaries and loss of mating responses in operated females. He added that reimplantation of pituitary fragments was followed by reappearance of sexual receptivity in hypophysectomized animals. Smith's colleague, Earl Engle, later described the induction of precocious puberty by pituitary treatment, and presented as an important part of his evidence descriptions of adult mating reactions in immature rodents (Engle, 1931).

During endocrinology's first 40 years, an especially active American center for research was the University of Chicago, where Frank R. Lillie was Chairman of the Department of Zoology and F. C. Koch headed the Department of Physiological Chemistry and Pharmacology. In 1917 Lillie had published his famous analysis of development of the freemartin (female twin of a normal male calf) which pointed to the conclusion that the fetal testis secretes a hormone normally contributing to the prenatal differentiation of masculine sex characters. In 1922 Lillie prepared the following list

of major problems in sexual development (Aberle and Corner, 1953).<sup>2</sup>

1. Influence of homologous and heterologous hormones in embryonic life. To what extent are sex characters reversible?
2. Sex modification in utero by other hormones or by antibodies.
3. Sexual modification after birth by hormones: castration, homologous and heterologous grafting, sex gland extracts, etc., including structure, function, and psyche.

This list is important in this history for two reasons: (1) It concentrates upon problems of sexual differentiation, and these have become a focus of research in behavioral endocrinology since the classic experiment on prenatal masculinization of female guinea pigs was published 37 years later by Phoenix, Goy, Gerall, and Young (1959). (2) It includes the term psyche, which signifies behavioral characteristics; and there is no doubt that at that time many endocrinologists sincerely expected behavioral studies would comprise an integral part of their discipline. This expectation was not limited to “founding fathers” in the United States. At the first meeting of the French Société d’Endocrinologie in 1939 one Professor Roussy asserted that hormones influence “le jeu des instincts, assui bien que [dans] les fonctions psychiques superieures.”

The reason that behavioral studies did not gain wide acceptance as endocrinology progressed is clearly exemplified in the chronology of certain events that developed at the University of Chicago and in other research centers in the United States, England, and Europe.

In the 1920s Koch and his staff of biochemists were busily preparing extracts of various glands, particularly the testis, with the aim of eventually obtaining its hormone in purified form. As each new extract became available it was rushed across campus to the Zoology Department where members of Lillie’s group could test its potency. The method of choice was called *bio-assay*, and involved measuring effects of the extract by administering it to animals that had been deprived of the gland of origin. Testicular extracts, for example, were assayed by determining the degree to which they would stimulate growth in the accessory glands of castrated rats. An additional approach was to discover whether castrated males given the extract would exhibit normal mating behavior.

One of Lillie’s first graduate students was Carl Moore whose first assignment was to attempt the production of a “guinea pig freemartin.” This Moore was unable to do. He next investigated Steinach’s claims that vasoligation induces rejuvenation in aged male animals, but all results were negative. There was no evidence of consistent change in testis morphology or

of increased androgen secretion. On the other hand, Moore was successful in replicating and extending certain other studies by Steinach, especially those indicating that gonadal hormones affect sexual behavior in adulthood.

All in all, beginning in 1919, Moore published a series of 11 articles with the generic title, “On the Physiological Properties of the Gonads as Controllers of Somatic and Psychic Characteristics.” His techniques included homologous and heterologous gonadal transplants into castrated animals of both sexes with apparent induction of normal or reversed mating patterns. Although such findings were of interest to Moore the zoologist, Moore the endocrinologist was distressed by the variability, or, as he saw it, the unreliability of behavioral responses to hormones. His ambivalent attitude is reflected in the following statement concerning testis secretions written in 1931.

After the stage of puberty, or masculine maturity, the loss of the hormone is less evident. It is clear in many specific cases, but less clear in others, that the instinct of mating is conditioned by male hormones, either directly or indirectly. To mention only two cases in which hormonal control is in doubt: We have often employed male guinea pigs, castrated at 30 days of age (prepubertal), as a means of detecting females in heat when actual mating was undesired; the mating instinct persists and male behavior persists for many months despite early castration. In the case of the white rat Stone learned that males castrated at an early age will copulate with females up to five or eight months. Castration, therefore, does not immediately and permanently destroy sexual inclinations, even in lower animals.

Moore’s experience with male accessories such as the epididymis and seminal vesicle had led him to expect that any character depending on testis hormone would respond to castration with rapid degenerative changes and total loss of function within a relatively short time. Furthermore the time course and completeness of these regressive effects would be essentially the same in all individuals. Male mating behavior did not follow any such “all-or-none” rule and was therefore suspect as an androgen-dependent trait. Having no training in behavioral methods Moore cannot be severely criticized for his failure to deal effectively with individual differences. However, had he more carefully studied the reports of C. P. Stone (whom he quotes) he would have seen that mating behavior in male rodents does undergo lawful and predictable changes as a consequence of castration in adulthood or before puberty.

The fact was, however, that behavior as a potential major topic of interest in endocrinology was already doomed for quite different reasons. Instead of expanding their initial interest in “psychic characters,” endocrinologists were becoming more and more involved in determining the chemical structure and physiological action of hormones. Behavior remained within the

pale only so long as it provided the most efficient method of bioassay. As soon as the estrogenic content of a glandular extract could be determined by its activity in a test tube there was no longer any need to go through the cumbersome process of administering it to spayed mice and recording presence or absence of sexual receptivity to males.

The general climate of opinion is reflected in the retrospective evaluation by Samuels (1958) to the effect that the impact of biochemistry turned endocrinology "from theory to science"; and we already have quoted Turner and Bagnara's statement that "the 1940's ushered in a period of biochemical expansion and enquiry unprecedented in the history of endocrinology." Any bright young endocrinologist receiving his training around that time could clearly see where his professional future lay so that he would scarcely waste time in experiments on behavior when there was so much to be learned about biochemistry and cellular biology.

There was, however, one young man who retained his identity as an endocrinologist and at the same time conducted experiments involving a behavioral variable. This exceptional individual was William C. Young who was a student of Carl Moore (and thus a "grand-student" of Frank Lillie). Young took his Ph.D. at the University of Chicago in 1927 by which time Moore had already begun to doubt the usefulness of behavioral measures in endocrinology. According to one account, Moore warned Young that "the behavior of animals was ... unordered by hormonal events, and unrelated to variables of significance to reproductive biology" (Goy, 1967). Nevertheless, Young held to the belief that some simple behavioral responses might be more reliable indicators of a hormonal state than any other index then available.

As Instructor and then Assistant Professor of Biology at Brown University he and two graduate students (Edward W. Dempsey and Hugh I. Myers) were able to demonstrate that in female guinea pigs the lordosis reflex can first be elicited at precisely that time in the cycle when the ripening ovarian follicle shows its initial stage of preovulatory growth. This in turn meant that by timing the onset of the behavioral responses Young could predict very accurately the time of ovulation.

As recalled years later by Dempsey (1968) this formidable project involved testing every animal every hour 24 hr per day for the presence or absence of the lordosis reflex. Literally hundreds of ovaries were sectioned, stained, and studied microscopically so that the number and size of follicles could be correlated with the individual's behavioral status.

Other experiments showed that spayed females could be brought into heat by injections of estradiol but only if the estrogen was followed at a suitable interval by one injection of progesterone. This finding was difficult

to reconcile with the contemporary belief that progesterone is secreted only by the corpus luteum which forms after ovulation has occurred. Reading his first formal paper before the American Association of Anatomists in 1935, the young graduate student Dempsey presented the conclusion that progesterone had to be present before ovulation because it was essential to the display of sexual receptivity. His hypothesis was flatly rejected by such eminent endocrinologists as Edgar Allen, George Corner (a codiscoverer of progesterone), Herbert Evans, and P. E. Smith. In a subsequent visit to the laboratory at Brown, Allen suggested to Young he would be well advised to give up behavior and return to his more promising early studies on physiology of the epididymis.

Some years later improved biochemical methods demonstrated the presence of small amounts of progesterone in the mature follicle before the occurrence of ovulation.

Up to this time Young was still using behavior as an indirect method of studying ovarian physiology, but his recognition of its importance expanded when he moved to the Yerkes Laboratories of Primate Biology in Orange Park, Florida, and began to examine reproductive biology of the chimpanzee in cooperation with R. M. Yerkes and other psychologists. Experimental reports published during his 4 years there reflect an increasing appreciation of the value of studying behavior in its own right, and the importance of considering social factors and individual differences in scientific investigation of complex behavior. This broadening of outlook was even more evident when, as Professor of Anatomy at the University of Kansas, Young maintained a very active research laboratory where graduate and postgraduate students (C. Phoenix, R. Goy, A. Gerall, M. Diamond, W. Riss, H. Feder, E. Valenstein, J. Grunt, and others) collaborated in the study of reproductive physiology and behavior. Young felt that his contact with psychologists altered his research. He once stated that without such interdisciplinary stimulation he would never have recognized the importance of experience in affecting behavioral reactions to hormones.

In 1963 Young became Chairman of the Department of Reproductive Physiology and Behavior at the Regional Primate Center near Portland, Oregon, and there he assembled a very strong group of young research workers dedicated to a systematic program of studies on the hormonal control of sex-related behavior in monkeys. W. C. Young is one of the most important figures in the history of behavioral endocrinology, (1) because of his many significant research contributions, (2) because of the breadth and integrative value of his theoretical writings, and (3) because of the many important contributions to the discipline which have

been and continue to be made by scientists he trained and inspired.

One additional category of endocrinologists who contributed indirectly to the development of behavioral studies consisted of medically oriented individuals with particular interests in relations of hormones to human pathology. For the most part these men and women belong on the outer fringes of this history, but one individual whose research is particularly relevant is Hans Selye. Born in Vienna, Selye studied in Prague, Paris, and Rome and then went to Johns Hopkins on a Rockefeller Fellowship. Eventually he became director of his own laboratory at the University of Montreal. Strongly influenced by Walter Cannon's studies on "emergency reactions" of the sympathetic system, Selye initiated a lifelong program of investigation of the body's reactions to stress. His concept of the "general adaptation syndrome" is well known, and an important aspect of the response pattern involves various endocrine changes, especially those of the adrenal glands.

Beginning in the 1950s Selye's theories and experiments drew the attention of psychologists who were interested in emotion and its relation to adaptive behavior. The results included a number of studies dealing with effects of stress in infancy on emotionality in adulthood, and, somewhat later, a separate line of research on the role of pituitary and adrenal hormones in learning motivated by stress.

#### **Contributions by Physiologists and Biologists Concerned with Animal Reproduction**

During the early part of the present century various individuals interested in theoretical and practical aspects of breeding in wild and domestic animals made important contributions that eventually would be incorporated into behavioral endocrinology. The decision to separate the work of these scientists from that of contemporary endocrinologists is arbitrary, but is based upon the impression that they were less interested in the biochemistry of hormones than in relations of the endocrine system to reproductive functions of the total organism including behavior. Furthermore, many of their studies dealt with effects of environmental stimuli on endocrine secretions which subsequently modify behavior.

Two particularly noteworthy individuals, both of them English, were W. Heape and F. H. A. Marshall. Heape's monographic treatise, *The "Sexual Season" of Mammals and the Relation of the "Pro-Oestrus" to Menstruation*, published in 1901, deserves mention because of its emphasis upon temporal relations between periodic changes in the ovaries, uterus, and vagina, and their close association with the behavioral characteristics of sexual receptivity to the male. In the same year,

Marshall, Professor of Physiology at Cambridge, described the estrous cycle of sheep, and pointed out that sexual receptivity in the ewe depends on the previous presence of a functional corpus luteum.

In 1925 Marshall and J. Hammond from the School of Agriculture at Cambridge studied sexual cycles in the rabbit and demonstrated that ovulation in this species does not occur spontaneously but depends on stimuli received by the doe during copulation (Hammond and Marshall, 1925). Clearly the nervous system must be involved, and Marshall was quick to perceive that the pituitary gland might be the first target of messages from the brain. In 1935 Marshall suggested that his graduate student, Geoffrey Harris, might find it worthwhile to try inducing ovulation in rabbits by applying electrical stimuli to the hypothalamus (Harris, 1972). The advice bore fruit (Harris, 1937) and was in part responsible for Harris' major influence on subsequent development of a new research area to become known as neuroendocrinology.

Marshall's writings are particularly enriched by his emphasis upon the role of exteroceptive factors in controlling reproductive rhythms and associated behavior. Another Cambridge zoologist, F. Fraser Darling, furthered this concept in his studies on social stimulation of reproductive cycles in birds that form breeding colonies (1938).

In 1913, the American ornithologist, Wallace Craig, made note of correlations between behavior and ovarian function in the common pigeon, pointing out that the female's ovulation depends on stimuli provided by the male's courtship and copulatory behavior. A few years later the biologist, Oscar Riddle, at the Carnegie Genetics Laboratory in Cold Springs Harbor, New York, reported that secretory activity of the pigeon's crop gland is facilitated by stimuli received from the mate, as well as from the nest and eggs. Riddle also showed that crop gland secretion depends on prolactin, the same pituitary hormone that is involved in milk production and maternal behavior of female mammals (Riddle and Bates, 1939). In 1935 Riddle observed the suppression of gonadal activity by prolactin, and suggested that sexual and parental activities may be antithetical in birds and mammals. A reverse correlation had already been predicted in 1916 by another American biologist, H. D. Goodale, on the basis of his finding that castrated roosters often become broody and incubate eggs laid by the hen. Goodale therefore suggested that testis hormone normally inhibits paternal tendencies in male fowl.

In many avian species seasonal reproduction is preceded and followed by extensive migratory flights, and the first experiment clearly establishing an involvement of gonadal hormones in such migrations was that of William Rowan, Professor of Zoology at the University of Alberta, whose classic study of the junco was

published in 1929. Briefly stated, Rowan's theory held that northward migration to the breeding grounds is dependent upon increase in gonadal hormone secretion. He suggested further that the underlying endocrine changes are controlled by seasonal changes in day length. Experimental attempts to validate the theory by artificially increasing or decreasing normal day length, castration, and hormone treatment were ingenious though results in terms of behavior were not clear cut. Nevertheless Rowan deserves a place in this history for even attempting to manipulate experimentally a complex sequence of phenomena through which environmental and endocrinological events are interrelated in the service of species survival.

Another biologist with special interest in environmental control of endocrine functions associated with reproduction was T. H. Bissonnette, who trained under Lillie at Chicago in the early 1920s, spent a year at Cambridge University in association with Marshall, and conducted most of his research as Professor of Zoology at Trinity College in Hartford, Connecticut. Over a number of years Bissonnette studied effects of light on the hypothalamus-pituitary-gonad complex in various animals, especially birds and ferrets. One of his important contributions was demonstration that as far as birds are concerned light of certain wavelengths is effective in stimulating the pituitary whereas other wavelengths are without influence.

#### **Contributions by Zoologists with Special Interests in Behavior**

During the first half of the present century a number of European zoologists were conducting experiments involving behavioral variables. For example, work begun in Germany during the 19th century by M. E. B. Schrader (1892) and others on the male frog's clasp reflex was continued by F. Edinger in 1913. Studies of sexual behavior in rats were prosecuted by H. Kun (1933) at the Institute in Vienna under Steinach's leadership. In France, A. Giar (1905) observed that the origins of "l'amour maternel" depend on stimuli received by lactating females while suckling their young; and in 1912 A. Pezard described effects of testicular extracts on the plumage and mating reactions of castrated fowl. In 1927 the Italian investigator, C. Ceni, reported that ovariectomy did not eliminate broodiness and maternal care in hens; but such behavior could be abolished by implantation of an ovary containing large follicles. This finding agreed with Riddle's hypothesis that the hormones responsible for mating behavior inhibit parental responses.

At the University of Edinburgh, B. P. Weisner and various colleagues, including N. M. Sheard, conducted an extensive program of elaborate and elegant studies on maternal behavior in rats. Their results, published

in 1933, indicated that nesting, nursing, and retrieving are controlled by a combination of stimulus factors associated with the young, and by hormones secreted by the pituitary gland.

At the same time that Frank Lillie and his associates were conducting their early experiments in endocrinology at the University of Chicago a vigorous research program dealing with social behavior of animals was underway on the same campus in the Whitman Laboratory of Experimental Zoology. The program was headed by W. C. Allee whose investigations of animal societies had already gained wide recognition. A senior worker in the Whitman Laboratory was L. V. Domm who, as a former student of Lillie, was particularly interested in the embryonic origins and hormonal control of morphological and behavioral sex differences.

In an attempt to modify such differences, Domm (1939) injected eggs of chickens with sex hormones at successive stages of incubation, and then noted the sexual characteristics of birds that had received the experimental treatment. In some instances partial sex reversal of morphological and behavioral characteristics was achieved. In association with David E. Davis, Domm also produced "intersexes" having a mixture of male and female traits (Davis and Domm, 1943).

Allee was especially interested in possible hormonal effects on social relations in animal groups. At different times with different students (e.g., N. Collias, E. Beeman, A. Guhl, H. Shoemaker, and C. Lutherman among others) he investigated the effects of thyroxine, testosterone, estrogen, and other hormones on aggression, dominance, and submissive behavior. Shoemaker was first to show that female canaries can be induced to sing if they are injected with testosterone. Subsequently at Amherst and then the University of Wisconsin, Collias continued to study hormonal effects on behavior of chickens, ring doves and pheasants (Collias, 1944). Guhl extended the original research on dominance and mating in chickens at the Kansas State Agricultural College (Guhl, 1944/1945). David E. Davis, a postdoctoral student with Domm, eventually became a major contributor to research on endocrine relations to population size. One of Davis' doctoral students at Pennsylvania State University, J. Vandenberg, later undertook investigation of social stimuli (produced by conspecifics) upon endocrine aspects of reproduction.

During the 1940s several field-oriented zoologists, exemplified by W. C. Bullough in England, and J. T. Emlen and F. W. Lorenz in America, made important studies of reproductive and sociosexual cycles of wild birds. Bullough's evidence (1945) on the wild starling was largely observational, but Emlen and Lorenz (1942) conducted field experiments to investigate, for example, effects of testosterone implants at different

times of year on the sexual and aggressive behavior within free-living flocks of quail.

During the 1930s at the University of Iowa, Emil Witschi (1932) and his associates in the Department of Zoology began a very extensive series of studies on the hormonal control of sexual differentiation of various vertebrate species. With one student, C. A. Pfeiffer, Witschi studied endocrine correlates of vaginal estrus, ovulation, and mating in rats. Pfeiffer's name is important to this history because he showed in 1935 that sex differences in gonadotropic functions of the rat pituitary can be traced to permanent effects exerted in the male by testis hormone secreted during the first few days after birth—a discovery of eventual significance for understanding of brain differences between the sexes.

Witschi's interest in pituitary secretion led him to the significant observation that it could be influenced by feedback stimuli aroused by an animal's own behavior. An experiment on reproduction in sparrows convinced Witschi (1935) that the behavior of the female during nest building and mating is "in some way very essential for the final hypophyseal stimulation and growth of the ovaries to final breeding condition." Like many fundamental concepts, this one had been partly anticipated but less clearly formulated by earlier workers, e.g., W. Craig in his 1913 descriptions of pigeon behavior mentioned above, and would much later be refined and tested experimentally by subsequent investigators (D. S. Lehrman in 1965 with the ring dove, and R. A. Hinde and E. Steel in 1966 with the canary).

In the 1940s a significant series of experiments on hormones involved in broody behavior of fowl was conducted at the University of Illinois Department of Animal Husbandry by A. V. Nalabandov and his associates. They demonstrated that roosters presented with newly hatched chicks usually killed them, but successive presentations coupled with successive injections of prolactin caused males eventually to adopt and care for foster young. Extending the earlier theorizing of Riddle regarding prolactin and parental behavior, Nalabandov (1945) hypothesized that prolactin inhibits release of gonadotropin which in turn decreases the production of testosterone by the testis. He concluded that testosterone deficit is essential to broodiness, a suggestion that fits in with Goodale's 1916 report of incubation of eggs by castrated roosters.

Another American biologist to display an early interest in hormonal effects on behavior was G. K. Noble. In 1928 he founded the Department of Experimental Biology at the American Museum of Natural History in New York City. From the beginning Noble envisaged a broad program of studies which included explanation of the role of hormones in social and sexual activities in a wide variety of vertebrates. He succeeded in completing some experiments with fishes (Noble,

Kumpf, and Billings, 1938), reptiles (Noble and Greenberg, 1941), and birds (Noble and Zitrin, 1942) but Noble's career was cut short by his premature death. He was succeeded in 1940 by the present writer as Chairman and Curator of the Department of Animal Behavior, who in turn was followed 6 years later by L. R. Aronson. For many years the department continued to function as an active center for research in behavioral endocrinology, and an initial training ground for future contributors to the field who would eventually receive degrees from various universities. A partial list of individuals working in the department at one time or another includes D. S. Lehrman, J. Rosenblatt, C. Diakow, A. Zitrin, B. Komisaruk, J. Stern, E. Tobach, and M. Cooper.

Any discussion of behavioral studies by zoologists would be incomplete without mention of the special field which has become known as *ethology*. The difficulty this presents, as far as the present history is concerned, is that although they have made many important contributions to behavioral endocrinology, ethologists did not begin experimental work with hormones until after the formative period had closed. A rare exception was W. M. S. Russell's use of human chorionic gonadotropin in 1954 to induce mating in *Xenopus*. Pioneers such as K. Lorenz and N. Tinbergen had relatively little to say about the importance of hormones. The first book on ethology was *The Study of Instinct* written by Tinbergen and published in 1951. The term "hormone" does not appear in the subject index and only three pages of text are devoted to consideration of hormonal effects on behavior. In contrast, *An Introduction to Animal Behavior* written by Tinbergen's student, A. Manning, and published in 1967 includes one full chapter "Hormones and Behavior." K. Immelmann's *Introduction to Ethology* published in 1980 and containing almost exactly the same number of pages as Tinbergen's volume devotes an entire 12-page section to hormones and behavior, and the subject index shows 28 page references to "hormone."

R. A. Hinde's comprehensive volume, *Animal Behavior: A Synthesis of Ethology and Comparative Psychology* (1970), and J. B. Hutchison's *Biological Determinants of Sexual Behavior* (1978) give eloquent testimony to modern ethologists' appreciation of the importance of hormones and to conduction of research in this area by experimental ethologists.

#### Recognition of Problems by Geneticists

Although the science of genetics became well established during the first half of this century geneticists in general conducted little research on behavior and their work is therefore not especially relevant to this history. An exception must be made in the case of C. R. Stock-

ard who, although not a professional geneticist, was interested in the possibility that behavioral and temperamental differences between various breeds of domestic dogs might be related to inherited differences in endocrine function.

With support from the National Research Council Stockard established a "Dog Farm" at Cornell University and part of the research conducted there involved studying effects of removal and replacement of various glands or their secretions on behavior in different dog breeds (Stockard, 1941). Measures of emotional or temperamental differences proved difficult to objectify and validate, and employment of Pavlovian conditioning methods (by W. T. James, a psychologist) yielded inconclusive results.

It was in fact not until nearly 20 years later that feasible methods for interrelating genotypic differences and behavioral response to hormones were worked out (e.g., Goy and Young, 1957; McGill, 1962); but during the modern era of behavioral endocrinology research along these lines has progressed steadily and fruitfully.

### Contributions by Neurophysiologists

We already have seen that 19th-century physiologists were convinced that hormones influence behavior by inducing changes in central neural mechanisms. In general, however, adequate experimental tests of these hypotheses awaited 20th-century improvements in neurophysiological techniques and instrumentation.

One of the first American investigators to undertake such study was Philip Bard, whose research began in the early 1920s while he was a graduate student in the Harvard Physiological Laboratory directed by Walter B. Cannon. Cannon's innovative theory of homeostasis had been widely received and he was at the time interested in studying relations between the sympathetic nervous system and the adrenal medulla; but Cannon was also concerned with possible effects of hormones on the brain. As a consequence Bard was encouraged to investigate central neural mechanisms responsible for the display of estrous behavior by female cats and guinea pigs. Currently available methods relied heavily on direct ablation which was somewhat imprecise, but Bard was able to show that removal of some brain regions, though not others, permanently abolished sexually receptive behavior even when hormonal conditions were appropriate for its occurrence (Bard, 1939).

Subsequent expansion of this program in Bard's own laboratories, first at Princeton and later at The Johns Hopkins University, included studies of behavioral reactions to estrogen in female rodents, rabbits, and cats after various ablations or transections of different parts of the CNS including the neocortex, thalamus,

hypothalamus, and ascending and descending spino-cerebral tracts. Students and senior colleagues who collaborated with Bard in these experiments at different times included C. McBrooks, C. D. Davis, W. S. Root, D. McK. Rioch, R. B. Bromiley, and H. W. Magoun. Among other findings, their research pointed to the hypothalamus as one brain region intimately involved in mediation of estrogenic effects on receptivity. This was of particular interest because, as explained earlier, research in F. H. A. Marshall's laboratory at Cambridge University had already indicated that the hypothalamus was in some fashion important to the occurrence of ovulation and therefore to the secretion of ovarian estrogen.

Another very active center of neurophysiological research destined to influence behavioral endocrinology was S. W. Ranson's Institute of Neurology at Northwestern University. Between 1930 and 1950 investigators at Northwestern pursued a vigorous program of studies employing the Horsley-Clark stereotaxic apparatus which permitted stimulation or destruction of localized subcortical areas without significant damage to overlying neural tissue. Using this instrument in 1938 C. Fisher, H. W. Magoun, and S. W. Ranson showed that mating behavior in female cats was permanently eliminated by lesions involving the anterior hypothalamus and preoptic areas. Two years later similar results were obtained in experiments on female guinea pigs by J. M. Brookhart, F. L. Dey, and S. W. Ranson.

It was gradually becoming clear that display of certain kinds of hormonally induced behavior depends on the functional integrity of specific brain regions, and at the same time it had long been suspected that the CNS exerts reciprocal control over the activity of at least some endocrine glands. In the course of an address delivered before the Royal and Imperial Society of Physicians and Surgeons in Vienna in 1912, Bernard Aschner, a colleague of Steinach, made the following observation.

Not only hypophysectomy, but even a mere wound in the base of the thalamencephalon leads to atrophy of the gonads in male and female dogs. The extent to which the hypothalamus can be regarded as a regulatory center for endocrine glands must therefore be decided after further research has been carried out. (quoted in Holweg, 1975)

The research called for by Aschner began years later as an outcome of interest in mechanisms affecting ovulation in mammals. Gradually its scope broadened until it gave rise to the new discipline of neuroendocrinology which finally achieved independent status during the 1960s (Meites, Donovan, and McCann, 1975) and was to exert widespread effects on subsequent developments in behavioral endocrinology.

### Concept of Neurosecretion and Contributions of Neuroendocrinologists

Thirty-five years after Aschner suggested that the “thalamencephalon” might regulate activity of some endocrine glands G. W. Harris proposed the theory that release of tropic hormones by the adenohypophysis is controlled by “neurohumors” that are secreted by nerve cells in specific hypothalamic areas and then transmitted to the anterior pituitary via the portal system.

A key notion in the theory was that a neuron might affect a gland, not simply by transmission of ordinary neural impulses, but indirectly by means of a blood-born chemical. This represented a departure from conventional thinking of endocrinologists accustomed to working exclusively with mammals, but was not a new idea to others whose research dealt with other organisms.

As early as 1917 S. Kopeć, a Polish zoologist reported a series of explantation experiments on caterpillars which led to the conclusion that their brain secretes a “pupation hormone” capable of inducing pupation in the entire body of the insect (Scharrer, 1975). By 1928 Ernst Scharrer, a student of Karl von Frisch at Munich, had demonstrated in a teleost fish the existence of special brain cells that respond to stimulation by releasing along their axons chemical material having many of the characteristics of hormones from endocrine gland cells. For many years the husband and wife team of Ernst and Berta Scharrer carried out a joint program with Ernst specializing in vertebrates and Berta invertebrates. Their work did not bear directly on behavior but had many important implications for subsequent developments in neuroendocrine relations essential to behavior.

For example, one of the most far-reaching implications embodied in the concept of neurohormones was the possibility that since their cells of origin are neural and lie within the CNS such secretions might serve as a “bridge” or link between environmental stimuli and secretory function in traditional endocrine glands. In other words, a visual, tactile, auditory, or olfactory stimulus which excited first a peripheral receptor and then its central connections might, if it eventually reached the hypothalamus, thereby gain access to the adenohypophysis and cause the release or inhibition of tropic hormones capable of influencing the gonads, adrenal cortex, thyroid, etc. Such a chain of events would help to explain how the courtship singing of a male bird could facilitate growth of eggs in the ovary of his mate, or odors of an estrous female might increase or maintain secretion of testosterone in males of the same species.

After this brief digression on neurosecretion we are in a position to deal more directly with contributions

of neuroendocrinologists to the early history of behavioral endocrinology.

About 1940 an American student at Cambridge University heard F. H. A. Marshall lecture on sexual behavior of animals and was strongly impressed by Marshall's comprehensive views regarding the interdependence of endocrine, neural, and environmental stimuli in the orchestration of events involved in reproduction. The student was Charles H. Sawyer who returned to conduct his doctoral research at Yale on the role of the enzyme cholinesterase in the development of swimming behavior in larval *Ambystoma*. This study revealed a remarkable correlation between (1) maturational changes in nervous system morphology (as previously described by G. E. Coghill), (2) rising concentrations of the enzyme, and (3) progressive development of locomotory movements leading to organized swimming behavior (Sawyer, 1943).

Sawyer's interest in correlations between neural, biochemical, and behavioral variables continued during a tour of appointments at Stanford, Duke, and finally, in 1951, the University of California at Los Angeles. There he joined H. W. Magoun (previously a member of Ranson's group at Northwestern), in establishing the Brain Research Institute that gradually emerged as an important American center for neuroendocrinology. During the following quarter of a century in collaboration with Magoun, J. Green, C. Barraclough, R. Gorski, and many others, Sawyer was directly or indirectly involved in an impressive series of studies, many of which were relevant to hormonal control of behavioral mechanisms.

Research in neuroendocrinology was, of course, proceeding at the same time in other laboratories, and as related to behavior the field has gradually come to involve at least three lines of investigation. Two focus on ways in which hormones affect the nervous system (1) with respect to its organization during early development, and (2) as regards its functional characteristics in adulthood. (3) The third problem deals with processes by which activity of the nervous system influences the secretion or nonsecretion of hormones by various endocrine glands.

To illustrate the kinds of research involved we will consider briefly two major techniques, one has to do with direct application of hormones to brain cells or nuclei, and the other with exposing the embryonic or neonatal brain to abnormal forms or amounts of hormonal stimulation.

### Direct Application of Hormones to the Brain

A special technique destined to influence theories of hormone action on behavior was initiated in 1949 when C. G. Kent and M. J. Liberman induced sexually receptive behavior in spayed hamsters by first priming

them with systemic estrogen treatment and then injecting various amounts of progesterone either intramuscularly or into lateral ventricles of the brain. Behavioral response to progesterone occurred most rapidly when the hormone was administered intracerebrally; furthermore, less progesterone was needed to produce this effect when it was injected into the brain than when it was administered systemically.

Certain investigations of the "locus of action" problem really belong at the beginning of the modern era, but will be mentioned here to illustrate the continuity of relevant research. A. E. Fisher in 1956 described the occurrence of sexual and parental responses in male rats after injection of sodium testosterone sulfate into the preoptic region; but the first conclusive proof of direct action of hormone *implants* on behavior was published in 1958 by G. W. Harris, R. P. Michael, and P. Scott. These workers inserted small amounts of crystalline estrogen into the anterior hypothalamus of spayed cats. A few days later the females began to exhibit normal mating behavior despite the fact that their vaginas remained in an anestrus condition and, therefore, the hormone presumably had not entered the general circulation. Subsequent studies by R. D. Lisk (1960) in Hisaw's laboratory at Harvard and J. M. Davidson at the University of California and Stanford (1966) as well as other workers in different institutions revealed that direct introduction of gonadal hormones into appropriate brain areas was capable of inducing sexual responses in gonadectomized females and males of at least some species of vertebrates.

Since the studies mentioned above were first reported more precise and sophisticated methods have been developed to administer and withdraw measured amounts of a particular hormone to restricted brain regions. These methods have been applied to mammals, birds, reptiles, amphibians, and fishes.

At the same time *in vitro* and *in vivo* examination of responses of single neurons to direct hormonal stimulation have advanced the understanding of intra- and intercellular events consequent to hormone action. As a result of such work progress is being made toward identification of both the locus and nature of hormonal effects that eventually modify behavior.

#### Organizational Effects of Hormones on the Brain

The history of this concept antedates by several decades the emergence of neuroendocrinology but it is properly discussed in this section because the point at issue centers on endocrine-nervous system interaction. Relevant studies eventually led to a new line of research in behavioral endocrinology and we shall devote some space to a chronological account of successive experiments because it provides an especially illuminating illustration of the way in which an important fact

is almost discovered, discovered but unrecognized, and finally rediscovered and firmly established as "disciplinary doctrine."

1922 F. R. Lillie posed the question (in his list of research topics mentioned earlier) as to whether homologous or heterologous gonadal hormones may affect differentiation in embryonic life, and the extent to which sex characters (including "psyche") may be reversible.

1930 C. Pfeiffer showed that if male rats are castrated at birth the pituitary functions in feminine fashion at adulthood. He erroneously concluded that sex differences in the gland itself are determined by presence or absence of testosterone during the neonatal period. Later work showed that the sex differences could be traced to the hypothalamus rather than to the pituitary.

1937 J. B. Hamilton and W. U. Gardner described anatomical masculinization of female offspring of rats which had received testosterone during pregnancy.

1937-1938 A. Raynaud described four experiments demonstrating that administration of androgen to pregnant rats induced "intersexuality" in the female offspring.

1938 V. Dantchakoff injected pregnant guinea pigs with testosterone and reported anatomical masculinization of the female fetuses. She also noted that when the masculinized females were given androgen in adulthood they exhibited male-like mounting behavior. What she did not know was that normal females of this species will mount without exposure to exogenous androgen.

1940 J. G. Wilson, W. C. Young, and J. B. Hamilton published an article titled "A Technique for Suppressing Development of Reproductive Function and Sensitivity to Estrogen in the Female Rat." Females were exposed to exogenous androgen during fetal development and also in the neonatal period. Behavior tests showed abolition of mating behavior even when the masculinized animals were given 10 times the amount of estrogen that regularly produced receptivity in normal females. This evidence was sufficient for formulation of the "organization theory" of hormonal action on the developing brain, but the point was somehow missed, only to be rediscovered 19 years later.

1943 G. van Wagenen and J. B. Hamilton injected pregnant rhesus monkeys with testosterone and thereby produced female offspring with male external genitalia.

1945 F. Beach reported that a female rat with congenital absence of the ovaries showed normal mating behavior when injected with estrogen and progesterone in adulthood. He concluded that gonadal hormones are not involved in organization of brain mechanisms for mating behavior. This was incorrect, but would

have been true had the interpretation been limited to females.

*1946* F. Beach and A. Holz castrated male rats at birth and found that when these animals were treated with testosterone in adulthood they exhibited vigorous copulatory responses to females but failed to ejaculate (presumably) because the penis remained infantile in size and intromission was rarely achieved. The conclusion was that postnatally secreted testis hormone is not essential to organization of CNS mechanisms for masculine sexual behavior. Reactions of neonatally castrated males to ovarian hormone were not examined. If this had been done it would have become apparent that such animals are highly responsive to estrogen and will show typical female mating reactions under its influence.

*1948* T. Martins and J. Valle described urination behavior of male dogs castrated in infancy and females given testosterone. To varying degrees the micturition behavior of males was feminized and that of females masculinized. Theoretical assessment of these findings is reflected in the following quotation.

Concerning the mechanisms of hormonal action it is necessary to determine whether, during the embryonic differentiation or early in the postnatal period, the hormones act as organizers inducing certain connections amongst special nervous centers; or whether they are only activators of genetically predetermined arcs and thus modify the thresholds of excitability. The functional effects here described indicate that the hormone possesses a selective action, a certain neurotropy which, during the developmental period, might well exert an organizing action, stimulating the development of specific centers or connections. However such interpretations are at present merely speculative.

*1954* C. A. Barraclough and J. H. Leatham administered testosterone propionate to female mice 5, 10, or 20 days after birth and tested their fertility at 3 months of age by leaving them with males until they became pregnant or for 100 days. Females receiving testosterone propionate at 5 days never became pregnant. Eight of twelve treated at 10 days, and 15 of 16 treated at 20 days eventually delivered litters. This study was important in showing a fairly rapid postnatal decrease in the negative effects of exogenous androgen on female reproductive function.

*1959* C. Phoenix, R. Goy, A. Gerall, and W. C. Young published their now classic article, "Organizing Action of Prenatally Administered Testosterone Propionate on the Tissues Mediating Mating Behavior in the Guinea Pig." Pregnant guinea pigs receiving testosterone propionate at specific stages of gestation produced female offspring with the following characteristics: (1) masculinization of external genitals, (2) failure to show feminine mating responses when supplied with large amounts of exogenous estradiol and

progesterone, (3) exhibition of vigorous male mating behavior when given testosterone in adulthood.

This experiment is the one generally cited as first establishing the "organizational" effects of hormones, and rightly so since it was the first in which experimental results on behavior were definitely linked to a clearly stated theory. The theory is that during special periods of development (which differ in different species) normally secreted testis hormone exerts permanent effects on organization of the male brain and thus influences the type of sexual behavior likely to appear in adulthood. Since the fetal or neonatal female produces no testis hormone her brain becomes organized in a different pattern and in adulthood she exhibits feminine behavior. Experimental exposure of females to exogenous testosterone during the appropriate stage of development induces masculine instead of feminine brain organization, thus suppressing female behavior and enhancing male behavior in adult life.

Since the original demonstration by Young's group of such effects in the guinea pig comparable phenomena have been shown to occur in other rodents, in dogs, sheep, monkeys, and also in birds, but discussion of the experiment involved would carry us well beyond the formative era of behavioral endocrinology.

### Neural Control of the Endocrine System

Technically speaking this section properly belongs within the province of neuroendocrinology, but history shows that responses of the endocrine glands to environmental stimulation were generally recognized long before the specialty of neuroendocrinology came into existence. We have noted already observations by the American ornithologists, Craig and Whitman, to the effect that the common pigeon previously isolated from others of her kind may be induced to lay eggs if she is exposed to a male or even if her head and neck are stroked repeatedly by her human caretaker. Mention also has been made of early studies by Rowan and others indicating a relation between seasonal onset of gonadal activity and annual changes in day length.

The basic mechanisms involved were first thoroughly investigated by neuroendocrinologists concerned with the control of ovulation in various species of mammals. Conclusive proof that induced and spontaneous ovulation depend on functional relations between neurosecretory cells in the brain and tropic-hormone-secreting cells in the anterior pituitary is rightly assigned, directly or indirectly, to efforts of early neuroendocrinologists such as G. W. Harris and Ernst and Berta Scharrer. The latter are included because of the basic importance of their work on neurosecretion.

However, once the basic mechanisms had been explicated the discovery was exploited by behaviorally oriented scientists in a manner that greatly expanded

our understanding of many correlations between environmental variables and behavior. Once it was comprehended that exteroceptive stimuli which influence brain function may thereby selectively modulate secretion of particular hormones which in turn could influence behavior, it became clear why specific patterns of stimulation so often evoked functionally adaptive patterns of hormonally influenced adaptive responses.

### **Contributions by Comparative and Physiological Psychologists**

The foregoing digression into neuroendocrinology carried this survey of the formative era somewhat beyond its announced temporal limits, but when attention focuses on the first contributions of psychology to development of behavioral endocrinology we return to an earlier period when endocrinology itself was only 20 years old. At that time experimental psychology had been in existence for nearly half a century and was in a unique position to capitalize on the use of hormones as independent variables in investigations of behavior. This was true partly by virtue of the philosophical and theoretical orientation of many contemporary psychologists, and partly because of methodological and technical expertise developed during several decades of laboratory experiments on human and animal behavior.

### **Philosophical and Theoretical Orientation**

Psychological theory in America during the early 1920s was strongly influenced by four trends, all of which combined to create a favorable atmosphere for initiation of experiments on hormonal control of behavior.

1. The first was a commitment to the importance of physiological explanations for subjective experience. This was evident in the popularity of so-called "physiological psychology," which had emerged from origins in psychophysics; and in the widespread conception of the brain as the basis of mental life.
2. The second trend was a growing popularity of the "behavioristic movement" which had been founded by John B. Watson in 1913. The major tenet of behaviorism was that the only proper subject of psychological study is overtly observable responses of animals and human beings. Private experiences such as sensations, mental images, emotions, and the like were rejected from the science of psychology unless they could be defined in terms of externally measurable reactions.
3. The third trend had begun in the 1890s when comparative psychology was imported primarily from England where it had arisen as a natural consequence of Darwin's insistence that natural selection favored mental as well as physical evolution. In empirically

oriented American psychology, philosophical speculation, and anecdotal approaches to the animal mind were gradually supplemented and then replaced by laboratory experiments on animal learning, memory, problem solving, sensory capacities, and the like. Behaviorism gave added impetus to the use of animal subjects in psychological experiments; and observation of other species stimulated new interest in the physiological bases of drive or motivation, as well as in functions of the nervous system and body chemistry in mediating instinctive or innately organized behavior patterns.

In short, by the early 1920s one segment of American psychology was theoretically and philosophically prepared to take advantage of recent advances in endocrinology in a manner quite different from that then practiced by endocrinologists themselves. For psychologists, primary concern centered on the analysis of behavior rather than analysis of hormones. The latter served as conveniently available experimental variables whose biochemical composition was of less significance than their effects on specific kinds of organized behavior typical of the individual animal or human.

### **Technical and Methodological Developments**

Interest in comparative psychology led to the establishment of animal laboratories in psychology departments at several American universities around the turn of the century. Edward L. Thorndike (1898) at Columbia and Robert M. Yerkes (1901) at Harvard pioneered in experimental investigations of "instinctive" behavior and habit formation (learning) in mice, cats, dogs, rats, and chickens. The movement spread rapidly to include other aspects of animal behavior and to involve psychologists in a growing number of institutions.

One consequence was that by 1920 psychologists had developed a modest but useful armamentarium of objective techniques and instruments for the description and measurement of behavior in a variety of species including fish, amphibians, reptiles, birds, and mammals from rats to monkeys. Special apparatus had been invented and standardized, and statistical methods appropriate to behavioral analysis had been validated. The latter point is of special significance, for it reflected the general recognition by psychologists of the importance of individual differences in behavior as mentioned above.

### **Pioneering Contributors between 1920 and 1930**

If one were to select a single psychologist whose contributions had the broadest and most lasting effects on research dealing with hormones and behavior the choice would have to fall on Robert Mearns Yerkes. This is true, not primarily because of Yerkes' own experimental

contributions, although they were considerable, but because of the influence he wielded over younger scientists as a founder of American comparative psychology and as a leader of the NRC Committee for Research in Problems of Sex to be described in a moment,

Beginning his publications on animal behavior in 1901, Yerkes was a broadly trained “psychobiologist” (as he preferred to be called) with 4 years of clinical work at the Boston Psychopathic Hospital and extensive experience in administration of the mental testing program conducted by the Surgeon General’s Office of the U.S. Army during World War I. In 1922 his national eminence was such that he was selected to assist in organization of a new National Research Council Committee for Research in Problems of Sex.

Initially conceived as a body to solve social problems of prostitution and venereal disease, the committee eventually compromised for less ambitious objectives, and under Yerkes’ chairmanship decided to support investigations into the physiology and psychology of reproduction. Membership of the NRC Committee in its early days included individuals already mentioned in this history, e.g., Walter Cannon, Frank Lillie, Willard Allen, George Corner, and Carl Moore.

Since several of these men were endocrinologists it was clear that the physiology of reproduction would be adequately covered and in fact it was for this committee that Lillie drew up the list of problems in sexual differentiation cited earlier in this account. At the same time Yerkes, as a psychologist, believed behavior should receive adequate emphasis, and to this end he asked an outside advisor, K. S. Lashley, to prepare a separate list of problems to include the “neurology and psychobiology of sex.” This was done, and chiefly as a result of Yerkes’ influence grants made by the committee often went to individuals interested in studying sexual behavior of animals or various problems related to human sexual psychology.

The importance of support distributed by this committee to development of hormone behavior research during the formative era of behavioral endocrinology has never been adequately recognized. Prior to World War II there were very few sources of research funds and none comparable in resources to present day national agencies such as the NSF, NIH, or NIMH. Individual grants of \$3000 for 1 year were regarded as generous, and it was especially difficult to obtain money for work on sexual behavior, one of the areas in which many psychologists were particularly interested. It may seem Victorian to younger readers, but as late as the 1940s, sexual behavior, even that of lower animals, was considered a “delicate” subject not only by the general public but also by many members of the scientific community. Therefore the decision of the Committee for Research in Problems of Sex to encour-

age investigations of copulation in rats (Stone, 1922), or frequency of orgasm in married women (Terman, 1939), was a courageous one that eventually opened the way for general expansion of research on effects of hormones on a very important category of behavior. There is no question that availability of grants from this one small agency with an annual budget of less than \$50,000 greatly facilitated development of behavioral endocrinology in America (for a history of the committee see Aberle and Corner (1953)).

Some impression of the breadth of the committee’s interests and effectiveness can be gained from a list of individuals mentioned in this history who received grants between 1922 and 1947. In alphabetical order they are as follows: E. Allen, L. R. Aronson, P. Bard, F. A. Beach, T. H. Bissonnette, W. B. Cannon, C. R. Carpenter, G. W. Corner, E. W. Dempsey, E. T. Engle, H. M. Evans, A. M. Guhl, A. C. Kinsey, F. C. Koch, K. S. Lashley, H. S. Liddell, F. R. Lillie, W. R. Miles, C. R. Moore, A. V. Nalabandov, G. K. Noble, G. N. Papanicolaou, C. A. Pfeiffer, S. W. Ranson, H. H. Shoemaker, J. R. Slonaker, P. E. Smith, C. R. Stockard, C. P. Stone, L. M. Terman, E. Witschi, and W. C. Young.

Another extremely important contribution of this same committee was sponsorship and underwriting of successive editions of *Sex and Internal Secretions* (1922, 1932, and 1961), an exceedingly influential survey of many facets of endocrinology including effects of hormones on sex-related behavior as discussed earlier in this account.

When Yerkes asked him to draw up a list of problems for research on the psychobiology of sex, K. S. Lashley was Assistant Professor of Psychology at the University of Minnesota where Yerkes previously had been chairman. Lashley’s scientific reputation rested on his innovative investigations of brain mechanisms and learning in rats and monkeys but what is not generally known is that as early as 1920 he was keenly aware of the importance of research on hormonal influences on behavior. At Minnesota he encouraged his students to pursue such work. For example, one undergraduate investigated maternal behavior in rats, and another, Josephine Ball (1926), examined possible effects of the estrous cycle on maze learning in rats. Lashley’s conviction that hormonal control of behavior offered a potentially important topic for research is reflected in a letter written to a prospective graduate student in 1920. Among various problems that might provide material for a doctoral thesis Lashley suggests the possibility of investigating

the effects of internal secretions upon specific reactions—for example, attempts to induce maternal behavior in virgin females by injection or implantation of a pregnant uterus. A great deal of preliminary work will be necessary... but

positive results would be pretty sure to make a man in psychology. (Beach, 1961)

The recipient of this letter was Calvin P. Stone, one of several experimental psychologists to initiate research on hormones and behavior between 1920 and 1930. Others were Curt P. Richter, Josephine Ball, and Carl J. Warden.

The most senior of this group was Richter, who studied animal psychology with Yerkes at Harvard and then moved to Johns Hopkins in 1919 to work under behaviorist John Watson. In addition to Watson, Richter was strongly influenced by his contacts with psychiatrist Adolph Meyer and other members of the medical faculty at Hopkins. His interests in hormones and behavior ranged widely, encompassing studies of general activity, thirst, food intake, diet selection, and behavior and mood cycles in women. Between 1922 and 1977 he published 86 papers dealing with endocrine functions.

In 1922, at the University of California, endocrinologists J. A. Long and H. M. Evans described the vaginal and behavioral estrous cycle in female rats, and 1 year later Richter's student, G. H. Wang (1923) published a monographic study of running-activity cycles in which the peak levels of activity coincided with vaginal estrus and sexual receptivity.

In this particular experiment general activity was quantified by maintaining the rats in revolving drums so that each revolution expressed exactly a given distance "traveled" by the animal. These original "activity wheels" were designed by Richter, whose first undergraduate training had been in structural engineering. Later the same apparatus was manufactured by a commercial supplier using Richter's specifications, and it has since been used in hundreds if not thousands of studies on "activity" of rats, mice, hamsters, gerbils, and many other small animals.

Another of Richter's students, Elaine Kinder, measured relations between estrous cycles and nest building in rats in 1927, and demonstrated a correlation, with maximal building during diestrus when the rat's body temperature is lowest. Much of Richter's most important work dealt with effects of hormones on body metabolism and the "homeostatic behavior" by which rats compensate for deficiencies associated with endocrine pathology. Some of his earliest studies dealt with increased ingestion of NaCl by adrenalectomized rats and of Ca solutions by rats deprived of the parathyroid glands.

Richter's comprehensive theoretical paper, "Animal Behavior and Internal Drives," published in 1927 in the *Quarterly Review of Biology*, exemplifies the interests and achievements of contemporary psychologists in behavioral endocrinology. Summarizing much of

his own research as well as that of other scientists, Richter illustrates various kinds of behavior affected by internal secretions, and concludes that the effects probably depend on hormonal stimulation of special centers in the brain and spinal cord.

Calvin Stone was one of several psychologists influenced by Karl Lashley to engage in research on hormones and behavior. Stone's doctoral research was published in 1923 with the title, "Experimental Studies of Two Important Factors Underlying Masculine Sexual Behavior: The Nervous System and the Internal Secretion of the Testis." After joining the faculty at Stanford University, Stone continued research in this area, and his experiments were models of careful design and thoughtful interpretation.

For example, Stone's 1927 study of mating in castrated rats involved repeated pre- and postoperative tests on large groups of subjects with separate frequency scores for each type of response, i.e., mounts-without-intromission, intromissions, and ejaculations. The exact time of occurrence of every response was recorded, and postoperative testing was continued for 6 months. The published report included measures of within-group variance, group averages, test-to-test reliability, statistical significance of differences, etc. As a result, the ways in which testosterone affects male copulatory performance were elucidated in detail which showed that different components in the total response are differentially dependent on the hormone.

Ejaculatory reflexes disappeared first after castration, and this was followed by loss of the ability to achieve intromission. Pursuit of the female and occasional mounting attempts might persist for half a year or longer. Finally, marked differences between individuals were evident; and total impotence could occur in one male 30 days after castration whereas another animal might retain the ability to intromit for several months.

The importance of variation between individuals was further emphasized by Stone's report in 1924 that some male and female rats occasionally display mating reactions characteristic of the opposite sex as well as those of their own without special treatment and without signs of endocrine abnormality. These observations (confirmed by the present writer in 1938) suggested the necessity of reinterpreting earlier reports by Steinach and by Moore, in which it was claimed that reversals of sex behavior had been produced by transplantation of heterologous gonads.

One of Stone's most intriguing experiments (1925) consisted of an unsuccessful attempt to induce maternal behavior in virgin rats by uniting them parabiotically with another female who was then mated and allowed to deliver and rear her litter. Certain chemicals, such as trypan blue, appeared in the virgin after

being injected into the lactating female, but there was no effect on maternity. However, an experiment published 47 years later indicated that Stone might have succeeded had he been able to establish adequate continuity of the circulatory systems of his paired females. In 1972 Terkel and Rosenblatt at the Institute of Animal Behavior, Rutgers University, maintained continuous exchange of blood between a parturient female and a virgin rat. They reported that the virgin exhibited nest building, retrieving, and nursing-posture responses to foster young.

Stone's concern with effects of hormones was not confined to the behavior of rats, but extended to psychological characteristics of humans as well. With one student he investigated the intellectual development of children exhibiting signs of *puberta praecox* (Doe-Kuhlman and Stone, 1927/1928). With another he compared the sociosexual attitudes in pre- and post-menarcheal girls of the same chronological age (Stone and Barker, 1934).

Stone's example and encouragement led several of his students to direct their own research along lines similar to his own. To mention only some of Stone's graduate students and their thesis research, we can list G. T. Avery (hormonal control of mating in guinea pigs, 1925), J. R. Slonaker (hormones and activity in rats, 1924), C. R. Carpenter (castration and mating in male pigeons, 1933), R. G. Barker (sex drive in male rats, 1935) and E. E. Anderson (estrous cycles and emotionality in rats, 1940).

Psychologists trained by Stone emulated his emphasis on methodological precision and detail in description and measurement of behavior. As late as 1950, N. Collias (already mentioned as a student in Allee's Chicago laboratory during the 1930s) published an extensive review of hormonal studies on bird behavior in which he made the following observation.

Carpenter is one of the very few authors who have tabulated frequency of sexual responses of birds during regular, time-limited, standardized observation periods in relation to endocrine studies. (Collias, 1950)

The study referred to was Carpenter's thesis work which involved observations on 46 castrated and 28 intact male pigeons for several months before and 6–9 months after operation.

Josephine Ball was an undergraduate student in Lashley's laboratory at Minnesota where she became interested in hormonal effects on behavior. As a graduate student in psychology at the University of California in Berkeley, Ball was Research Assistant to the endocrinologist Herbert Evans, and in 1929 she utilized rats from his colony to conduct her doctoral thesis, "Measurement of Sexual Behavior in Male Rats." This exemplary normative study involved 18 months

of observation on 61 subjects in repeated tests under standardized conditions. Results were presented in terms of mean values, standard deviations, odd–even reliabilities, etc., on each of several independently scored items of behavior.

Ball eventually located at the Department of Embryology, Carnegie Institute of Washington in Baltimore, where she did some work with Richter, but was associated primarily with the Director of the Institute, Carl Hartman, an expert in ovarian physiology and embryology, and later with his successor, George Corner, codiscoverer of progestagenic hormone of the corpus luteum. Both Hartman and Corner encouraged Ball in her behavioral experiments which included the first demonstration of sexual receptivity induced in ovariectomized monkeys by estrin injections (1936), and the additional discovery that in this species estrogen-induced receptivity is inhibited by progesterone (1941).

Psychologists in the 1920s were as interested as their modern successors in animal learning; and at that time they were just becoming aware of studies on conditioning which had been in progress for a number of years in the physiological laboratory of I. P. Pavlov in Leningrad. Pavlov and his colleagues had already reported that conditioned reflexes in dogs can be affected by secretions of the thyroid, parathyroid, adrenals, and gonads. In 1926 the American psychologist, Howard Liddell,<sup>3</sup> returned from postdoctoral study in Leningrad to establish at Cornell University a special laboratory where he proceeded to conduct a long series of experiments on conditioning and "experimental neurosis" in sheep and goats as affected by various hormones.

Experiments on various types of learning in rats and other animals were carried out by psychologists in a number of laboratories. As reviewed by the present writer in 1948, conditioning and maze learning were the favored behavioral measures, and experimental manipulations involved the removal of various glands including the gonads, thyroid, thymus, parathyroid, and adrenal. Investigation also was directed toward possible effects of administering different sorts of extracts, emulsions, and even desiccated forms of those glands which could be utilized in such a manner. Although scores of experiments were published the results ranged from ambiguous to negative. In fact no clear progress was made until after 1950 when investigation first focused on fear-motivated avoidance conditioning and its relation to the pituitary–adrenal system. In expanded form this work has proven fruitful (Levine, 1968) but belongs outside the formative era.

Beginning with 19th-century physiologists who studied effects of castration on mating in frogs, a common way of "explaining" a hormone's effects on behavior had been to say that it altered this or that

“drive,” or that it increased or decreased a specific type of “motivation.” An unfortunate circularity was involved because strength of drive was simply inferred from observations of the consummatory response. One of the first serious attempts to circumvent this difficulty and to study more directly the effects of hormones on animal motivation was undertaken by experimental psychologists.

During 1925 in the Psychology Department at Columbia University, C. J. Warden initiated a comprehensive series of experimental investigations to measure and compare “the reaction tendencies or drives associated with hunger, thirst, maternity, various sex conditions, etc.” This was to be accomplished through use of what became known as “The Columbia Obstruction Apparatus,” a device in which the animal was required to cross an electrically charged grid before entering the goal chamber which contained the appropriate incentive.

L. H. Warner used the obstruction technique in 1927 to compare the number of crossings made by female rats to a male at different stages of the vaginal estrous cycle (first described by Long and Evans only 5 years earlier). Warner found that crossings were most frequent when the vaginas were cornified, indicating stimulation by ovarian hormone. Two years later H. W. Nissen employed the same technique in his Ph.D. research which was published with the title, “The Effects of Gonadectomy, Vasotomy [*sic*] and Orchic Extracts on the Sex Behavior of the White Rat.” He discovered that castration reduced a male’s tendency to cross to a spayed female which had been injected with placental extract, but subsequent injection of testicular extract to the male had no remedial effects (Nissen, 1929).

Psychologists of the 1920s were scarcely qualified to prepare extracts of placental or testicular tissue, but the group at Columbia was generously assisted by the endocrinologist, G. N. Papanicolaou, whose research with Stockard on the guinea pig’s estrous cycle has already been mentioned. His laboratory was conveniently situated near Columbia at the Cornell Medical School. Papanicolaou was particularly supportive of two Columbia graduate students, John and Georgene Seward, whom he encouraged and financed in their research on sexual and parental behavior in guinea pigs which was continued when they moved to Connecticut College where no animal laboratory was available so that the animals had to be housed in a garage and behavior tests were run on the kitchen floor (Seward and Seward, 1940).

#### **Continued Research by Psychologists in 1930s and 1940s**

Research by Richter, Stone, and others plus a succession of their students was continued throughout the next two decades. A particularly important event was

the opening of the Yale Laboratories of Primate Biology in Orange Park, Florida, in 1930. Yerkes became Director of this institution while retaining his appointment as Professor of Psychology at Yale. This arrangement facilitated cooperative research with Edgar Allen whose endocrinological laboratory was located in the Yale Medical School in New Haven.

Yerkes not only initiated research on hormonal control of behavior in chimpanzees, but also encouraged and supported field studies of New World monkeys by Stone’s former graduate student, Clarence Ray Carpenter. During 1934 and 1935, with support from the NRC Committee for Research in Problems of Sex, Carpenter investigated the sociosexual behavior of free-living howler monkeys and spider monkeys in Central America, giving special attention to effects of females’ sex cycles on group behavior. This was followed by a study of the wild gibbon in Thailand (Siam). Carpenter subsequently published two reports on behavior of the rhesus monkey colony which had been established on Santiago Island just off the coast of Cuba. Each of Carpenter’s investigations, which today are regarded as classics in field primatology, included detailed records of sexual activities, group structure, and dominance, and the effects on such phenomena of seasonal and cyclic hormonal changes in males and females (Carpenter, 1942).

At Orange Park Yerkes and a graduate student from Yale, James Elder, made the first detailed study of estrus, sexual receptivity, and mating in normally cycling chimpanzees (Yerkes and Elder, 1936); and Allen’s laboratory in New Haven provided complementary data concerning cycles of estrogen excretion in the same animals. Another Yale psychology student, O. L. Tinklepaugh (1933), described the onset of sex cycles in pubescent females and its modification during pregnancy, together with behavioral observation of male–female interactions under each of these conditions. In 1938 Elder demonstrated that female chimpanzees injected with estrogen showed swelling of the sexual skin and became strongly attractive to males.

Yerkes himself was especially interested in dominance relations between sexes, and in 1940 he and his student, Meredith Crawford, reported that within established pairs the male usually is dominant, but yields to the female when she is in sexual swelling. W. C. Young arrived at the laboratories in 1939 and, working with various psychologists, proceeded to investigate further the relations of ovarian physiology to social and sexual behavior. With W. D. Orbison, in 1944, Young repeated and extended the original observations of Yerkes and Elder, using more refined behavioral measures and a larger population of chimpanzees.

Two additional psychologists working under Yerkes’ supervision studied the effects of exogenous gonadal

hormones on chimpanzee behavior. George Clark and Herbert Birch in 1945 described sexual behavior of a prepubertally castrated male before and after administration of testosterone. In addition they investigated changes in dominance status of ovariectomized females treated with estrogen.

The present writer's involvement in research on hormones and behavior began in 1936 during a postdoctoral year in the laboratory of K. S. Lashley at Harvard University. It originated as the result of conversation with graduate students in the adjoining endocrinology laboratory of F. L. Hisaw. The author's experiments had shown that male rats ceased copulating after infliction of large lesions to the cerebral cortex, and Hisaw's students pointed out that brain injury might indirectly reduce testosterone secretion by altering pituitary function. If so, loss of sexual responsiveness could have been due to functional castration. To check this possibility brain-operated rats that had ceased to mate were injected with large amounts of testosterone, and some then began to copulate.

Postmortem examination of the testes and accessory glands proved that testosterone secretion had not been impaired despite the cerebral lesions. Therefore it appeared that supplying very large amounts of exogenous androgen had somehow reversed a behavioral deficit caused by CNS injury. This serendipitous finding launched a career of research on the hormonal control of behavior.

Writers of history are hardly qualified to evaluate effects of their own activities on the trends they are describing, but it may not be inappropriate at least to mention a few high points and to name a few associates with whom one worked.

At the American Museum of Natural History Lester Aronson was a valued colleague and fellow worker who subsequently studied hormonal and nervous control of reproductive behavior in fish, rats, and cats. Arthur Zitrin was a Masters Degree student originally associated with G. K. Noble on studies of hormonal induction of precocious sexual responses in chicks who worked with the author on endocrine and neural mechanisms mediating mating in cats.

Having taken no formal training in endocrinology the writer audited an undergraduate course taught at New York University by Robert Gaunt, and was shocked by the absence of any reference to behavioral effects of hormones. At Professor Gaunt's invitation the author prepared and delivered one lecture to the class on the importance of behavioral research. This lecture was expanded to constitute a term paper and subsequently developed into the book, *Hormones and Behavior*, published in 1948.

As a professor at Yale University the author directed the thesis research of several graduate students who

conducted independent studies in behavioral endocrinology after receiving their degrees. Three of these were Charles Rogers, Richard Whalen, and Ronald Rabedeau. At this time Rollin Denniston was a Postdoctoral Fellow in the laboratory.

In 20 years at the University of California, Berkeley, the author enjoyed and profited from collaborative research with a large number of graduate students and postdoctoral fellows. Those who continued to work in the hormone-behavior field after leaving Berkeley included the following, named in order of their tenure in the author's laboratory: Knut Larsson (postdoctoral), Thomas McGill (postdoctoral), Julian Davidson (postdoctoral), Gordon Bermant (postdoctoral), Stephen Glickman (postdoctoral), Giorgio Bignami (postdoctoral), Lynwood Clemens, Burney LeBoeuf, Benjamin Sachs, Donald Dewsbury (postdoctoral), Norman Adler, Gray Eaton, Leonore Tiefer, Dale Wise, Ralph Noble, and Richard Doty (postdoctoral).

So swiftly does one academic generation succeed another that several of the writer's former students now have students of their own who have completed their training and are actively contributing to advancement of the field. As indicated in detail above the same sort of proliferation has, of course, occurred among the "academic descendants" of many other psychologists and nonpsychologists mentioned in this history.

Second- and third-generation contributors cannot be discussed individually in this account because their number is too large, but it is necessary to mention the contributions of one psychologist whose work, beginning in the 1950s, exerted a strong influence on subsequent developments in behavioral endocrinology.

Daniel S. Lehrman was a graduate student of T. C. Schneirla and received his Ph.D. from New York University. Before that, as an undergraduate, he had conducted research with G. K. Noble in the Department of Experimental Biology (later the Department of Animal Behavior) at the American Museum of Natural History. There Lehrman was first exposed to problems involving hormonal control of behavior although at the time he seems not to have been involved in research on such problems. In 1954, as an Assistant Professor at Rutgers University, Lehrman began a major series of experiments in a laboratory he created in an old loft building in Newark. Seventeen years later, still at Rutgers, he founded and became Director of the Institute of Animal Behavior.

Joined subsequently by another student of Schneirla, Jay Rosenblatt, Lehrman conducted a comprehensive program of studies in serial patterns of behavior in the ring dove. His choice of birds unquestionably was related to his lifelong interest and professional competence in ornithology. One of his major contributions was strong emphasis upon the interrelatedness of envi-

ronmental stimulation, hormonal state, and behavioral responses. In harmony with the classic observations of Whitman, Craig, Marshall, and others Lehrman stressed the importance of social stimuli from conspecifics which influence the secretion of hormones that in turn modulate behavior of the recipient.

Though his initial interest in hormones probably dated from contacts with Noble, Lehrman was influenced principally by Schneirla's profound theoretical approach to comparative psychology. To Schneirla he owed his comprehensive appreciation of behavior as an integrated, functionally adaptive process; but the lucidity and persuasiveness of his theoretical writings was Lehrman's own. His empirical research was highly original and fundamentally important, but Lehrman's most enduring contribution eventually may prove to have been the stimulation and leadership he provided for the many graduate students and postdoctoral fellows who came under his influence.

To this point our account of hormone-behavior research by psychologists has concentrated on the work of American investigators. The emphasis is obligatory. It reflects the fact that in other countries psychology had not become a biological science to the same extent as it had in the United States. Furthermore, relatively few British or European psychologists were involved in physiological investigations with animals. Nevertheless, there were individual workers in other countries with a strong interest in hormonal effects on behavior.

A conspicuous example was Andre Soularic, Professor of Psychophysiology at the University of Paris. Another, less widely published but highly original investigator was E. Wulff Rasmussen, psychologist at the University of Oslo. In 1943 Soularic replicated and extended certain of Richter's earlier studies of diet selection by rats deprived of various hormones. Later he engaged in a lengthy series of experiments on sexual behavior of rats which led him to conclude that although the ejaculatory response is hormonally controlled, copulation without ejaculation depends on neural mechanisms that are not under androgenic influence. Rasmussen studied activity cycles in rats and conducted several experiments on sex drive and hormones in mice using his own version of the obstruction method.

Although it is out of chronological order, mention should be made at this point of the fact that Soularic and to a lesser extent Rasmussen strongly influenced the scientific development of Knut Larsson of the University of Goteburg. Larsson's first publication in 1956 dealt with mating in rats, and though it did not involve hormonal variables, nearly all of his prolific research in the next 20 years falls in the area of behavioral endocrinology as does much of that conducted by Swedish students he subsequently trained at Goteburg.

During the period under consideration the interest of psychologists in effects of hormones was not confined to behavior of lower animals. Many correlational and a few experimental studies involved use of human subjects. For example several investigations were conducted to assess the role of epinephrine (adrenalin) in human emotions (reviewed in Landis and Hunt, 1932). Results were variable but clearly definable changes in emotional state were not reliably induced by administration of the hormone. At most, subjects reported what was termed "cold emotion," i.e., feeling "as if" they were afraid or "as if" they were angry. In a few instances genuine emotions were experienced but in the absence of a perceived reason for an emotional experience the majority of participants simply described their physiological symptoms.

There were a few attempts to assess the psychological effects of castration or congenital hypogonadism in men (reviewed in Beach, 1948), but reports of reduced "libido" or impotence did not constitute new evidence. A topic of considerable interest was the possible effect of hormonal changes associated with menstruation on intellectual and emotional functions. Although numerous interview and questionnaire studies were conducted the results were generally inconclusive or contradictory (Beach, 1948).

The most that can be said for psychological studies of human behavior and hormones during this historical period is that psychologists were alert to and interested in the basic problems concerned although there was no real progress toward their solution.

### Major Trends during the Modern Era

If the formative period of behavioral endocrinology is assumed to have drawn to a close or to have merged gradually into the modern era between 1950 and 1960 it will be obvious to anyone familiar with the field that many important changes have occurred since that time. As one whose research spanned much of the formative era and all of the modern up to this point, the present writer believes that at least five major developments have occurred.

#### 1. Increase in the Quantity of Research

The most obvious and easily documented change has been a positively accelerated increase in the sheer mass of published research which in turn reflects recruitment of a growing number of investigators interested in problems of behavioral endocrinology. Many of the new workers have been students or "grandstudents" of individuals whose work during the formative era already has been described.

One indication of the growth of research output over the last 2 or 3 decades may be the number of books

and articles that have appeared in that time. The first book-length summary of the field, *Hormones and Behavior* (Beach, 1948), stood virtually alone for nearly 20 years after its publication. Then, in the next decade no less than eight books appeared. Listing only those with obviously relevant titles the record is as follows.

- 1967 *Hormones and Behavior: An Enduring Problem in Psychology*, R. E. Whalen, ed. Collection of 19 previously published experimental articles. Van Nostrand, Princeton, N.J.
- 1968 *Endocrinology and Human Behavior*, R. P. Michael, ed. Results of a symposium with 18 separate papers and accompanying discussion. Oxford Univ. Press, London/New York.
- 1972 *Hormones and Behavior*, S. Levine, ed. Multi-authored volume of 8 chapters. Academic Press, New York.
- 1974 *Hormones and Sexual Behavior*, S. Carter, ed. Collection of 36 previously printed articles with editorial comments. Dowden, Hutchinson, and Ross, Stroudsburg, Pa.
- 1975 *Hormonal Correlates of Behavior*, B. F. Eleftheriou and R. L. Sprott, eds. A two-volume, 800-page compendium of 20 chapters by 25 authors. Encyclopedic approach organized primarily according to different types of behavior. Plenum, New York.
- 1976 *Hormones and Psychopathology*, E. J. Sachar, ed. Twenty-four papers presented at an Annual Meeting of the American Psychopathological Association. Raven Press, New York.
- 1978 *An Introduction to Behavioral Endocrinology*, A. I. Leshner. The first formal textbook. Oxford Univ. Press, London/New York.
- 1979 *Sex, Hormones and Behaviour*, E. J. Sachar, ed. Fourteen papers presented at the Ciba Foundation Symposium 62 (new series).

These eight books by no means represent the only reviews and syntheses published in the modern era. Hormonal effects on behavior have been exhaustively discussed in various handbooks, annual review series, conference symposia, etc., too numerous to mention separately.

Another item deserving mention is the establishment of new journals intended partly or entirely to accommodate publication of research on behavioral endocrinology. *Physiology and Behavior* began publication in 1966 under the editorship of M. Wayner and since that time approximately 25% of its articles have dealt with relations between endocrine and behavioral variables. In 1969 the present writer together with R. E. Whalen and J. M. Davidson established the journal *Hormones and Behavior* to publish research appropri-

ate to this title. In its first 8 years approximately 300 experiments were reported in this journal alone. The journal *Psychoneuroendocrinology*, under the editorship of R. P. Michael, first appeared in 1975, and since then has consistently included in its contents numerous experimental and clinical studies pertaining to behavioral endocrinology.

The fact that only the titles of books and journals published in English have been mentioned should not obscure the importance of contributions from other countries and publication in other languages. The greatest increase in numbers of workers and consequently in the volume of publications has occurred in the United States, but behavioral endocrinology has not developed in this country alone. Space does not allow the identification of individual contributors, or even each specialized research center, but basic and original research programs have flourished at different universities and institutes in, among other countries, England, France, East and West Germany, Holland, Hungary, and Sweden. Exchange of postdoctoral students and collaborative research by scientists in different countries have given an international dimension to developments during the modern era.

## 2. Advances in Correlational Studies

During predisciplinary and formative periods the vast majority of studies consisted primarily of establishing correlations between endocrine and behavioral variables. Correlational investigation has continued in the modern era but several changes have taken place. The most obvious of these involve (a) increase in the number and variety of species studied, (b) expansion of the types of behavioral patterns investigated, (c) multiplication in the number of hormones examined, and particularly, (d) major advances in techniques for measuring and controlling endocrine variables.

Prior to 1950–1960, especially in the United States, at least half and possibly 75–80% of all experiments on hormones and behavior employed as subjects the laboratory rat, mouse, or guinea pig. Since that time much more diverse types of mammals, especially non-human primates, have received increasing investigation. Nonmammalian vertebrates were used as early as the predisciplinary era but changes since the 1950s have included initiation of systematic, long-term programs of research on hormonal control of behavior in birds, fishes, reptiles, and, to some extent, amphibians. Beginning approximately in 1960 a few laboratories undertook programmatic study of effects of hormones and neurohormones on insect behavior.

Research by 19th- and early 20th-century pioneer workers concentrated heavily on mating behavior, and sexual activity has continued to receive strong emphasis, but other categories of behavior have come in for

increasing amounts of attention. Some of these have been (a) nonreproductive social behavior, (b) ingestive behavior such as eating and drinking, (c) seasonally cyclic behavior including migration and hibernation, (d) behavior involving parent-offspring interactions, (e) stress or fear-motivated behavior, and (f) behavior related to learning and memory.

The first types of hormones to be studied from the behavioral point of view were those of the gonads, thyroid, and adrenal glands. Pituitary secretions initially received attention only in terms of their tropic effects on "peripheral" epithelial glands. In more recent times behavioral studies have been extended to cover possible direct effects of adenohypophyseal hormones, behavioral responses to insulin, melatonin, oxytocin, vasopressin, hypothalamic neurohormones, and several of the prostaglandins.

Correlational studies during the latter part of the modern era have become increasingly precise and informative by virtue of technical advances in biochemistry and neuroendocrinology. A few illustrative examples are the radioimmunoassay methods of detecting and measuring amounts of different hormones in plasma and body tissues, antihormones which can temporarily and reversibly suppress action of specific hormones, analogs of natural hormones with particular structural units removed, radioactively tagged hormones whose transport through the organism can be charted and timed, and a variety of methods permitting localized application to or withdrawal of hormones from selected target areas within the organism.

### 3. Explication of Mechanisms of Hormonal Action

This has been an area of particularly vigorous and intensive research. We have seen that from the earliest predisciplinary studies research workers have hypothesized that hormonal effects on behavior are mediated by way of the CNS and this conviction remains dominant to the present day. During the modern era attempts to advance our understanding of nervous system involvement in hormone-behavior relations have focused on three major issues, i.e., (a) hormonal control of differentiation of organization of neural mechanisms, (b) localization of points of hormonal action within the CNS, and (c) effects of behaviorally significant hormones on neuronal metabolism.

We have already discussed the "organizational" role of hormones in sufficient detail for historical purposes and it suffices here to add that research on prenatal and neonatal effects of hormones has become an extremely popular topic for investigation in the course of the modern period. Scores if not hundreds of experiments have been conducted using various mammals (rodents, carnivores, ungulates, and primates) plus birds, and examining many different types of behavior.

Attempts to localize sites of hormonal action have benefited greatly from some of the technical advances mentioned earlier including availability of labeled hormones whose uptake can be traced, sensitive methods of analysis by means of which hormone concentration in different brain regions can be measured, delicate recording devices to detect localized changes in functional activity induced by controlled variation in hormonal stimulation, and specialized instruments and materials allowing direct and reversible application of hormones to selected brain or cord nuclei and tracts.

It is self-evident that a hormone can influence neural control of behavior only by affecting the function of individual nerve cells, and some behaviorally oriented investigators have become intensively engaged in study of cellular metabolism as affected by peptide and steroid hormones, especially in those areas of the brain already implicated in the control of hormonally influenced behavior. Such specialized problems as those involving receptor molecules in the cytoplasm, transport to the nucleus, modification of RNA and mRNA formation, and consequent modification of protein synthesis by the cell have recently become matters of considerable importance to behavioral endocrinologists.

### 4. Adaptive Values of Hormonal Control of Behavior

While some scientists have been looking inward in search of mediating mechanisms, others have shown increasing concern for analysis of the ways in which hormonal control of behavior affects relations between organism and environment and promotes adaptive functions in the individual and the species. Presumably such control has evolved because it increases capacities for survival of the individual and perpetuation of the species. The question then becomes one of determining the ways in which effective adaptation to environmental demands and opportunities is influenced by endocrine factors.

Research related to such enquiries can be divided roughly into two categories. The first encompasses studies of environmental variables that affect the individual's endocrine status, and we have seen that questions of this general nature had been raised in the early 1900s by students of reproductive function including F. H. A. Marshall, F. Darling, W. Heape, and later by W. Rowan and T. Bissonnette. The second category comprises investigations centering on the interactional or reciprocal aspects of behavioral sequences involving two or more conspecific individuals.

Modern era advances in research relevant to the first category have included increased understanding of "homeostatic" behavior which facilitates adjustment to changing environmental demands, e.g., relations between low external temperature, thyroid secretion, and nest building, or seasonal changes in day length as

associated with hormonally controlled increase in food intake and fat deposition prior to hibernation or to migration. Perhaps the most striking findings have been those relating to effects of the "social" environment on endocrine conditions which in turn affect behavior. Included in this subcategory would be demonstration of suppressed testosterone secretion in socially subordinate male monkeys, retardation of sexual maturation in female rodents exposed to pheromones produced by mature females in a crowded population, and elevated corticosteroid or cortisol levels associated with acute psychological stress.

In passing it should be mentioned that awareness of the importance of external influences on secretion of behaviorally relevant hormones has been partly the cause and partly the result of a steady increase in the number of observations and experiments carried out on free-living animals, or under a combination of field and laboratory conditions. During the formative period with few exceptions nearly all field research was conducted by zoologists, but more recently specialists in other disciplines such as psychology have become more and more deeply involved in such investigations.

Logically related to issues of adaptive function has been the increased emphasis on involvement of hormones in psychological processes such as perception, learning, memory, and emotional response. In particular, the pituitary-adrenocortical system has been shown to be intimately involved in promotion and retention of learned responses evoked by punishment or fear. Conversely, learned fear or anxiety is now known to be capable of inducing clear-cut "stress" responses including sudden increase in the release of adrenal corticoids as well as producing reduction in brain peptides such as antidiuretic hormone.

The second category of research relating to adaptive functions of hormonally controlled behavioral reactions is well illustrated by studies of prolonged sequences or temporal patterns of behavior in which successive phases are influenced by different hormones. Such situations frequently characterize protracted social interaction between individuals of the same species, e.g., between mates or between parents and offspring. A simplified example follows.

Stimuli derived from courtship by a male bird can induce ovarian development and estrogen secretion in a female partner. Ovarian hormones induce the female to build a nest. Stimuli received from the nest and perhaps from the male incite further hormonal changes eventually causing the female to ovulate. After oviposition, stimuli derived from the eggs suppress ovarian activity and evoke prolactin secretion which leads to incubation. As long as prolactin levels remain high, pituitary gonadotropins are low and the ovaries remain

inactive. Once incubation is completed and the young become independent they no longer provide the stimulus for prolactin secretion. Reduction of prolactin relieves the pituitary from inhibition and allows resumption of secretion of gonadotropic hormones which in turn stimulate estrogen secretion and prepare the female for another cycle of mating, nesting, egg-laying, and incubation.

The foregoing description is not intended to accurately or completely represent the reproductive pattern of any one species although each of the relationships involved is known to obtain in some species. The point of the synthetic account is to illustrate the type of relationships which are currently being studied by some behavioral endocrinologists with special concern for the functional, adaptive significance of hormone-behavior relations.

### 5. Attenuation of Disciplinary Distinctions

Throughout the main body of this article developments during the formative era could be organized with reasonable accuracy in terms of the disciplinary affiliations of the investigators involved. One of the most prominent changes in the course of the modern era has been a progressive diminution in the relevance of such distinctions. Today it is always difficult and often impossible to distinguish between the research of zoologists, psychologists, neuroendocrinologists, pharmacologists, and members of still other disciplines when that research centers on problems of hormones and behavior.

Scientists with their doctorates in psychology study development of progesterone receptors in neurons of the rat hypothalamus while other investigators initially trained in pharmacology invent elegant behavioral measures of sexual motivation in the estrous female. These developments appear to represent more than a mere borrowing of techniques by one discipline from another. Instead they seem to reflect progress toward recognition of common goals and shared theoretical interests. If such indeed is the case, behavioral endocrinology may well be a discipline *in statu nascendi*.

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## Notes

1. Including, according to some accounts, no less a personage than Sigmund Freud.
2. The list was drawn up for the National Research Council Committee for Research in Problems of Sex which is described later.
3. Liddell is included here as a psychologist although his original training and graduate degree were in physiology. When the Cornell Medical Faculty was moved from Ithaca, Liddell remained and became a member of the faculty of the Department of Psychology.

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